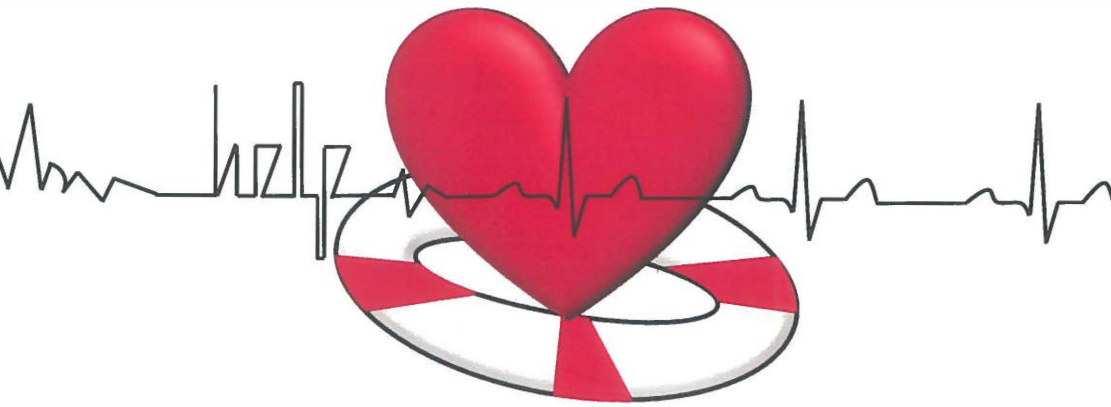
Molecular Remodeling in Atrial Fibrillation: Protective Roles of Small HSPs



Lei Ke

Molecular Remodeling in Atrial Fibrillation: Protective Roles of Small HSPs

Ke, Lei

To my dearest parents
献给我的家人

STELLINGEN

behorend bij het proefschrift

Molecular Remodeling in Atrial Fibrillation: Protective Roles of Small HSPs

Lei Ke

1. Calpain activation represents a key factor underlying cardiac troponin degradation, myolysis and contractile dysfunction in atrial fibrillation. (*This thesis*)

2. The Rho pathway plays a prime role in the reduction of calcium transients and induction of atrial fibrillation. (*This thesis*)

3. The chaperone-like activity of small heat shock proteins is not of prime importance for their protective role on molecular remodeling in atrial fibrillation. (*This thesis*)

4. Every disease is a physician. (*Irish Proverb*)

5. Maintenance of sinus rhythm (rhythm control) appears preferable for the treatment of atrial fibrillation. However, studies to date have failed to demonstrate tangible advantages of rhythm control. (*Dobrev and Nattel Cardiovascular Research (2011) 89,689-691*)

6. I am sure that even the simplest cell is still more delicate than a super-computer. (*Zhai Zhonghe, translated from "我确信哪怕一个最简单的细胞，也比迄今为止设计出的任何智能电脑更精巧" -翟中和*)

7. The purpose of original research is to make a meaningful contribution to knowledge, rather than to identify niches where "original" work can be done.

8. Imitate it until you have it.

9. While Dutch claim to be "gezellig", this is certainly not substantiated by their behavior in trains, where they spread their luggage on the nearby seats, thus preventing anybody to sit next to them.

10. Life moves pretty fast. If you don't stop and look around once in a while, you could miss it. (*Ferris Bueller*)

11. 人在高潮时，享受掌声；人在低潮时，享受人生。(*吴宗宪*) Life's ups provide one with self-esteem, but life's downs provide one with the cherished love from friends and family.

12. No one realizes how beautiful it is to travel until he comes home and rests his head on his old, familiar pillow. (*Lin Yutang*)

The research described in this thesis was conducted at the Department of Cell Biology, Section of Radiation & Stress Cell Biology, University Medical Center Groningen, University of Groningen, the Netherlands.

This research project was financially supported by the Dutch Organization for Scientific Research (NWO), the Dutch Heart Foundation and Ubbo Emmius Foundation.

The printing of this thesis was financially supported by: University of Groningen, the Dutch Heart Foundation, Graduate School of Medical Sciences, Department of Radiation & Stress Cell Biology and IonOptix Europe.

ISBN: 978-90-367-5147-6

Page layout: Ke, Lei and Gan, Xialing

Cover: Gan, Xialing

Printed by: Ridderprint BV
Yangke Printing Co.

cmb.

RIJKSUNIVERSITEIT GRONINGEN

Molecular Remodeling in Atrial Fibrillation: Protective Roles of Small HSPs

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 9 januari 2012
om 12.45 uur

door

Lei Ke
geboren op 11 oktober 1977
te Chongqing, China

Promotores:	Prof. dr. H.H. Kampinga Prof. dr. R.H. Henning
Copromotor:	Dr. B.J.J.M. Brundel
Beoordelingscommissie:	Prof. dr. U. Schotten Prof. dr. R. Tanguay Prof. dr. I.C. van Gelder

TABLE OF CONTENTS

Promotores:	Prof. dr. H.H. Kampinga Prof. dr. R.H. Henning
Copromotor:	Dr. B.J.J.M. Brundel

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Part of the research described in this thesis was supported by a grant of the Dutch Heart Foundation (DHF-2007B217; DHF-2009B024).

CHAPTER 1

General Introduction and Scope of Thesis

1. Atrial Fibrillation

Atrial Fibrillation (AF) is the most common sustained and progressive clinical arrythmia and a significant contributor to cardiovascular morbidity and mortality (1). In the healthy heart, the normal electrical impulses generated by auto-rhythmic cells of the sinus node travel through the two upper chambers (atria) of the heart and reach towards the ventricles via the purkinje fibers through the atrio-ventricular node (AV node). At rest, the normal impulses lead to the heartbeats at a typical rate of about 60-70 beats per minute (bpm), whereas they can reach up to 180-200 bpm during heavy exercise. In AF, the normal electrical impulses are overwhelmed by disorganized and much faster electrical impulses (400-600 bpm) that derive from the atria and pulmonary veins, leading to atrial quivering or fibrillating instead of atrial contraction. Thanks to the limited conductive capacity of AV node, only about a third of these irregular impulses are transferred to the ventricles that generate the increased heartbeat of about 100-200 bpm (1).

The irregular heartbeats may occur in episodes lasting from minutes to weeks, or they could occur all the time for years. The natural tendency of AF is to become more persistent over time (2). In many cases, AF may be asymptomatic and a patient for the first time becomes aware from a routine physical examination or electrocardiogram (ECG). AF usually results in symptoms related to a rapid heartbeat, like light-headedness, palpitations, chest discomfort. Occasionally, rapid and irregular heartbeats may also be perceived as angina, shortness of breath or edema. Furthermore with longer AF existence, it develops to other severe cardiovascular problems, such as chronic heart failure (CHF), stroke, thromboemboli or infarction (1, 3).

In some cases, AF, as a second condition, is associated with a variety of primary conditions, such as hypertension, cardiac surgery, pericarditis, congestive/coronary heart failure, congenital heart disease, pneumonia or other acute pulmonary disease. These primary conditions may be the cause of the AF (3, 4).

In about 30% of AF, patients are absent with any clinical or ECG findings of other

cardiovascular disease, cardiac abnormalities or related pulmonary disease. In these cases, it is called "lone AF". Some argued that it might be caused by other risk factors, including alcohol abuse, obesity, metabolic syndrome, psychological stress or even genetic factors (5-7).

Based on episode timing and termination, AF can be divided into four categories: 1) *first detected AF*, in which all AF patients are initially included; 2) *Paroxysmal AF* occurring in episodes that terminate spontaneously and generally lasts less than or equal to 7 days (mostly less than 24 hours). 3) If AF lasts more than 7 days, it is unlikely to terminate spontaneously but can be converted to sinus rhythm by cardioversion. This stage is called *persistent AF*. 4) If cardioversion is unsuccessful or deemed unnecessary, AF is ongoing for a long time and called as *permanent AF* (3). Persistent and permanent AF are also referred to as chronic AF (CAF) (3).

2. Basic Mechanisms of AF Initiation

Over the past decade, great advance has been made in the understanding of mechanisms for AF initiation. At the present time, two hypotheses predominate to explain AF initiation (1, 8):

a) ectopic activity: While the rate of atrial cell firing is faster than that of sinus node, ectopic activity occurs. Pulmonary veins (PVs) may also act as sources of ectopic activity for AF (9). Any factors that contribute to premature depolarization (the accelerated slope of phase 4) would cause the increased spontaneous rate and therefore leads to ectopic activity.

b) Reentry: In this mechanism, wavelet reentry produces either single or multiple atrial circuits that may each meander, interact or extinguish. To better understand this mechanism, the useful concept "wavelength" is worthwhile to be mentioned. The "wavelength" is defined as the distance traveled by an impulse within one effective refractory period (ERP) and so determined by the product of ERP duration and

conduction speed at which the impulse travels through the atrial tissue. For reentry to be maintained, the impulse must traverse all points of the entire circuit only if it falls outside the ERP at each point. Otherwise the wavelet reentry would extinguish. The wavelength approximates the shortest path length of reentry and therefore determines the size of maintainable reentry circuit.

When the wavelength of the electrical impulse is relatively greater, the impulse travels slowly enough through the whole atrial tissue in a manner of single circular way. Multiple circuit reentry works in a similar way to single circuit re-entry, but in this case a shorter wavelength will require the shorter minimum path length of individual circuits, and therefore allows more circuits to occur at the same time. In this manner, the likelihood for spontaneous termination of the multiple circuit reentry would be reduced and thus AF would be easier maintained.

In conclusion, any factors relating to the mechanisms described above would contribute to AF initiation and the subsequent remodeling would furthermore augment the probability of AF maintenance and progression.

3. Atrial Remodeling

The concept "atrial remodeling" defines any change in atrial structure or function that promotes atrial arrhythmogenesis. The concept was prompted by early clinical observations: many patients were seen to transit from increasingly frequent and longer paroxysmal AF to persistent AF, until permanent AF eventually ensued. The longer AF persists, the more difficult it becomes to maintain sinus rhythm after cardioversion (10,11). The changes in atrial tissue can be divided into two principal forms: tachycardia-induced remodeling and structural remodeling.

3.1. Tachycardia-induced remodeling

In Wijffels' pioneering work, he found that AF itself was the cause of the abnormal

electrophysiologic properties, which could in turn promote the recurrence and maintenance of AF. The phenomenon is also referred to as electrical remodeling and described as "AF begets AF" (12). The rationale for the self-promoting nature of AF is that tachycardia induces a reduction of ERP and the reduced ERP leads to the decreased wavelength promoting wavelet reentry and the recurrence/maintenance of AF.

To understand the rationale, it is important to look at the ionic basis of cardiac action potential. At the physiological status, the membrane potential of atrial myocytes remains around –70 mV at rest. Once the cell is electrically stimulated, the opening of the fast Na^+ channel and the rapid influx of Na^+ into cells trigger a depolarization of the cell membrane potential up to +30 mV. When the cell membrane potential is positive, the fast Na^+ channel is inactivated. At that moment, the transient net outward K^+ ions through the transient outward K^+ channels (I_{to}) cause a brief downward deflection of the action potential. In the meanwhile, the inward movement of Ca^{2+} through L-type calcium channels (I_{CaL}), balanced with the outward movement of the slow delayed rectifier potassium channels (I_{Ks}), leads to the plateau stage of cardiac action potential. The sodium-calcium exchanger current ($I_{Na\text{-}Ca}$) and sodium/potassium pump current ($I_{Na\text{-}K}$) also play minor roles during this phase. When L-type Ca^{2+} channels close, the slow delayed rectifier potassium channels (I_{Ks}) is still open and ensure negative change in membrane potential. Under this circumstance, the rapid delayed rectifier potassium channels (I_{Kr}) are open and cause the cell to reploarize (13).

3.1.1. Tachycardia-induced changes in Ca^{2+} handling

With the onset of tachycardia, the rapid and irregular atrial rates substantially causes intracellar Ca^{2+} overload in atrial myocytes (14). Due to the fact that Ca^{2+} overload is cytotoxic, atrial myoctyes respond by reducing the amount of Ca^{2+} entering into cells via L-type Ca^{2+} channels. The influx of Ca^{2+} is a major contributor to the "plateau" phase of cardiac action potential, and thus the tachycardia-induced reduction of Ca^{2+} transient

leads to decreased action potential duration (APD) favoring AF recurrence and maintenance (8).

In the early stage of response, the reduction of Ca^{2+} transient occurs because of functional inactivation of L type Ca^{2+} channels. The functional inactivation is dependent on the voltage of cell membrane potential and Ca^{2+} concentration in atrial myocytes (15). In this manner, APD can be affected as quickly as 7 minutes after the induction of tachycardia in humans (16).

When tachycardia sustains, Ca^{2+} transient is persistently reduced via activation of the Ca(2+)-dependent calmodulin-calcineurin-NFAT system to cause transcriptional downregulation of calcium L-type channel protein subunits (17). For the α1 subunit of calcium L-type channel protein, the downregulation was observed both at the mRNA and protein level (18-20) and at posttranslational steps such as protein dephosphorylation and proteolysis (21, 22). In addition, the use of calpain inhibitor in an *in vitro* model of AF attenuated the breakdown of L-type Ca^{2+} channel protein (23). In a rabbit model, researchers have also detected the reduced expression of all three β-subunits, which also suggests an important role of L-type Ca^{2+} channel protein in tachycardia-induced remodeling (24).

3.1.2. Tachycardia-induced changes in other ion channels

Transient outward K^+ current (I_{to}) has been seen to decrease both in animal models (24,25) and in AF patients (26,27). Decreased I_{to} is associated with the observation that both mRNA and protein expression of its subunit Kv4.3 showed the reduction (18). The breakdown of protein subunit Kv4.3 may be mediated by the increased calpain activity (22,28). The decreased expression of Kv4.3 mRNA may be contributed by Ca^{2+}-dependent protein phosphatase calcineurin (29), since an increased activity of calcineurin was reported in human AF (30). I_{to} counters with inward Na^+ current during the phase 1 of action potential, and thus the downregulation of I_{to} increases the action

potential amplitude. However, the functional implication of I_{to} downregulation is unclear in AF setting (8).

The delayed-rectifier current I_{kr} and I_{ks} are not changed in experimental model of atrial tachycardia (25), and the information from AF patients is lacking.

3.2. Atrial structural remodeling

If tachycardia-induced remodeling progresses, the atrial structural remodeling will occur resembling hibernation, which creates a further promoting substrate for AF perpetuation (22,31,32). Hibernation is a form of tissue adaptation which resembles ischemic hibernation and is defined as the ability of the myocytes to turn into a non-functional phenotype by degradation of the myofibril structure (myolysis), which leads to contractile dysfunction (31,33-35). Other characteristics are redistribution of nuclear chromatin, loss of sarcoplasmic reticulum, increase in abnormal shaped mitochondria and increase in glycogen accumulation. Interestingly, no profound imbalance in energy status during chronic AF was observed (36). Degenerative changes are not observed, but instead atrial myocytes shift towards a fetal phenotype (dedifferentiation). Cell viability will, however, be maintained for a prolonged period of time, thus ensuring tissue integrity (but not tissue functionality) (34). Consequently, myolysis is found in patients with persistent AF and not in patients with exclusively displaying paroxysmal forms of the arrhythmia (22), suggesting a role for myolysis in the progression of AF.

Atrial structural remodeling also happens as a result of other cardiovascular diseases underlying AF, like heart failure. In Morillo's pioneering study, AF-associated ultrastructural changes in atrial myocytes were first reported (37). These structural changes of atrial myocytes resemble the alterations in ventrical myocytes occurring after ischemia (ischemic hibernation) (38,39). Myocytes with hibernation caused by tachycardia or AF are thought to remain viable, ensuring tissue integrity, but loose their contractile function, which may explain the function of myocytes is still impaired even

after cardioversion of AF (33-35).

In animal models, AF-associated structural remodeling includes increase in cell size (hypertrophy), myolysis (loss of myofibrils), changes in quantity and localization of structural cellular proteins, homogeneous distribution of nuclear chromatin as in fetal cells (dedifferentiation), altered connexin expression, fragmentation of sarcoplasmic reticulum, change in mitochodrial shape and size, accumulation of glycogen (31,40).

In AF patients, similar changes were found (22,41,42), however some degenerative features were also observed. The myocytes from patients with AF and atrial dilation showed signs of DNA cleavage and programmed cell death (43). These broader structural changes than in animal models was suggested to relate to the older age and/or associated heart diseases (40).

The exact mechanisms underlying the various structural alterations relating to AF are not fully known yet. The electrical remodeling is completely recovered within a few days even after prolonged AF (32,44). On the contrary, myolysis, the most prominent change in AF, was still seen in many atrial myocytes even after 16 weeks of sinus rhythm, suggesting AF-related structural changes are difficult to be reversible (41). The activation of cystein protease seems one of the principal components linking AF-related electrical remodeling and structural remodeling (22,23).

4. Heat shock proteins in AF

4.1. Heat shock proteins and cardioprection

It has been recognized that AF induces cardiomyocyte stress. Since induction of the heat shock response provides cytoprotective effects that may be beneficial in a variety of acute diseases (45), including major cardiac disorders (46), it was hypothesized that a heat shock response might also be benificial in AF. There are at least five main heat shock protein (HSP) families, small Hsp (HSPB), Hsp40 (DNAJ), HSP60 (HSPD), Hsp70 (HSPA), and Hsp90 (HSPC), each with several family members, (specific) co-factors in

various cellular localizations and distinct and overlapping functions (47,48). These highly conserved proteins have important roles in protein folding, trafficking and cell signaling (47,49,50).

A substantial literature described the induction of HSPA1A (Hsp70) by ischemia and inverse correlation between expression of HSPA1A and infarct size in animal models (46). In addition, overexpression of HSPA1A offers a cytoprotective effects in cardiac myocytes subjected to simulated ischemia, improves myocardial function in animal models with ischemia, preserves metabolic functional recovery, and reduces infarct size after ischemia/reperfusion (46). Besides HSPA1A, also HSPB1 (Hsp27) and HSPB5 (alpha B-crystallin) can protect primary cardiomyocytes against ischemic damage (46).

Although the precise mechanisms are insufficiently understood, HSPs are thought to mediate these cardioprotective effects in ischemic diseases through their biological functions as molecular chaperones. *In vitro* overexpression of the small Hsp family member HSPB1 enhances alpha-actinin and F-actin stability and recovery after disruption (51,52). The stress-inducible HSPA1A binds and protects the microtubule network and therefore limits myofibril disruption after ischemic stress in myocardium (53). These observations suggest that HSPB1 and/or HSPA1A might have a protective effect in AF-induced structural remodeling.

4.2. Heat shock proteins and AF

The conventional therapeutic approach, the use of antiarrhythmic drugs, has been proven to have side effects on the electrophysiology of the ventricles, in such a way that they can paradoxically cause arrhythmic disorders and increase mortality (54). Recently, a detailed summary of pharmacological approaches to prevent atrial remodeling and their efficacy were extensively discussed (55). Rather than preventing arrhythmia, another approach could be used to make cells resistant to AF-stress and prevent the early triggers for remodeling and therefore more permanent structural remodeling. Hereto, HSPs might come into play. Many HSP can act as molecular chaperones and hereby were shown to be

able to protect cells against various forms of proteotoxic damage. Indeed, HSPs were found to play a protective role in various cardiovascular diseases (Table 1).

Table 1. Major stress proteins and their protective roles in cardiac diseases.

Stress protein	Previous family name	Approx. number of human family members	Protective Member	Cardiac Disease	References
HspA	Hsp70	15*	HspA1A	Ischemic heart disease	(56,57)
DNAJ	Hsp40	>40*	DNAJA3	Dilated cardiomyopathy	(58)
HspB	Small Hsp	10	HspB1 (Hsp27)	Atrial Fibrillation	(59)
				Ischemic heart disease	(60,61)
			HspB5 (αBCrystallin)	(Dilated) Cardiomyopathy	(62,63)
			HspB6 (Hsp20)	Ischemic heart disease	(64)
HspD	Hsp60	8*	HspD1 (Hsp60)	Heart failure	(65)
HspC	Hsp90	8*	HspCA (Hsp90)	Ischemic heart disease	(66)

*= includes possible pseudogenes.

Two studies reported elevated expression of mitochondrial HSPs, specifically HSPD1 (67), HSPE1 and mortalin (HSPA9B) in atrial tissue from patients with AF (68). HSPD1 and HSPE1 form a mitochondrial chaperonin complex, and a previous study has shown that their increased expression exerts a protective effect against injury when cardiac myocytes are submitted to ischaemia (69). Also in AF, the up-regulation of these mitochondrial chaperonins (HSPD1 and HSPE1) in combination or individually may play a role in the endogenous response to AF and by maintaining mitochondrial integrity and capacity for ATP generation, they could form crucial factors in determining survival of

cardiac myocytes during AF. However, besides induction of HSPD1, HSPE1 and HSPA9B (67,68) also a reduction of HSPD1 (70) has been reported as response to AF. Thus, whether mitochondrial Hsp indeed play a role in AF remains to be determined. To date, no systematic studies to ectopically up-regulate their expression before or during AF have been performed to more conclusively address this hypothesis.

Both Rammos et al. (71) and Mandal et al. (72) studied HSPA1A expression levels in atrial tissue of patients in sinus rhythm undergoing cardiac surgery. Both studies consistently showed a lower incidence of post-operative AF in patients with higher atrial HSPA1A expression levels. A recent study in atrial fibroblasts from rats showed HSPA1A, induced by a general heat shock, could be involved in the prevention of angiotensin II mediated atrial fibrosis and increased atrial vulnerability to extrastimuli (73). Whereas this suggest that HSPA1A may be involved in prevention of AF initiation, the role of HSPA1A on AF promotion (measured as duration of induced AF) and AF vulnerability (percentage of sites at which AF was induced by single premature stimuli) were not studied. In fact, studies from our own lab with an *in vitro* AF model using paced mouse cardiomyocytes suggested that HSPA1A did not prevent structural remodeling. Rather, these data suggested a role for small HSPB proteins in preventing AF-mediated structural remodeling (59). Therefore the focus of this thesis will be on the role of HSPB proteins in AF

4.3. Small heat shock proteins

The family of small heat shock proteins (HSPB family) consists of at least 10 members and they are expressed in various human tissues (Table 2). HSPB members are defined by a conserved C-terminal domain of ~90 amino acids (the α-crystallin domain) flanked by a variable length N-terminal arm and a more conserved C-terminal extension. It is thought at least some HSPB members assemble into homo-and/or heterogeneous oligomeric complexes, which dissociate into smaller multimers (74). Another important characteristic is that some HSPB members can be phosphorylated at certain sites to

regulate their activity, maybe by regulating the oligomeric state of the proteins.

Table 2: Characteristics of small HSPB members (74).

Gene Name	Protein Name	Alternative Name	Sequence Identity	Molecular Size (kDa)	Heat Inducibility	Expression in Heart	Other tissue expression
HSPB1	HSPB1	Hsp25, HSP27, HSP28	100%	22.783	Yes	+++	Uterus, skin, platelets, brain, kidney, some tumor cells
HSPB2	HSPB2	MKBP	36%	20.233	No	+	Skeletal muscle
HSPB3	HSPB3	HSPL27	23%	16.966		+	Skeletal muscle
HSPB4	HSPB4	αA-crystallin, CRYAA, CRYA1	36%	19.909	No	-	Lens of eye, spleen
HSPB5	HSPB5	αB-crystallin, CRYAB, CRYA2	38%	20.159	Yes	++++	Lens of eye, vascular wall cells, lung, kidney, brain, some tumor cells
HSPB6	HSPB6	Hsp20, p20	34%	17.136	No	++	Skeletal muscle, stomach, liver, lung, kidney, platelet
HSPB7	HSPB7	cvHsp	20%	18.611	?	++++++	Skeletal muscle
HSPB8	HSPB8	Hsp22, H11	34%	21.604	Yes	++	Skeletal muscle, stomach, liver, lung, kidney, brain
HSPB9	HSPB9	FLJ27437	19%	17.486	?	-	Testis
HSPB10	HSPB10	ODF1	17%	28.366	?	-	Testis

There are at least three, not mutually exclusive, biochemical functions that have been ascribed to small Hsp. First, both cell free and cellular data have suggested that (some) HSPB members can act as ATP-independent chaperones believed to prevent irreversible protein aggregation. Oligomeric dynamics seems to be crucial for such HSPB activities (75). HSPB-clients may next be processed (renaturation or degradation) through

cooperation with ATP-dependent chaperones (76,77). As such, stress-induced protein damage that could include AF-mediated remodeling, may be prevented. Second, several HSPB members were shown to be associated with cytoskeletal proteins in a phosphorylation dependent manner (78,79). This may on one hand result in stabilization of certain cytoskeletal structures (78,79), which - in turn - may be associated with increased resistance myofilament proteins against AF. On the other hand, some HSPB members were suggested to be involved in actin-reorganization (79), suggesting they may assist in recovery from AF-mediated myolysis. Finally, although biochemically poorly understood, HSPB members may inhibit the action of proteases (80) and as such may interfere with the cystein protease that seems to be activated upon AF.

4.4. The diverse members of the HSPB family: functional similarities and divergence

Beside the role of HSPB1 in assisting in refolding and/or targeting denatured proteins, another well characterized function of HSPB1 is its ability to interact with several cytoskeletal components, including actin, intermediate filaments, and microtubules (78). In muscle tissue, HSPB1 is found in association with sarcomeres and suggested to be cardioprotective (59).

HSPB2 is associated specifically with myotonic dystrophy protein kinase (DMPK) and also have been called a DMPK-binding protein (MKBP), suggesting its important role in muscle maintenance (81). During muscle differentiation, the 150-kDa complexes consisting of both HSPB2 and HSPB3 are induced, which suggest both HSPB3 and HSPB2 to play a role in muscle development (82). During ischemia, HSPB2 can translocate from cytosol to myofibrillar fraction, Z line, which suggests HSPB2 may offer protection against ischemia/reperfusion injury via binding to certain myofibrillar structure (83).

HSPB4 (α A-crystallin) and HSBP5 (α B-crystallin) are both strongly expressed in the eye lens, where they together maintain lens transparency. Both proteins indeed have

chaperone-like activity in cell free systems (84) and in living cells (85) which may (in part) explain their function in the eye lens. In the heart, HSPB5 co-localizes in the I-band and M-line region of myofibrils in cardiomyocytes (86). HSPB5 is known to bind and stabilize myofibrillar proteins, including actin, desmin and titin (87,88). Some HSPB5 mutants have been reported to be involved in myofibrillar myopathy and loss of ischemic protection (89). Like HSPB1, HSPB5 also plays an important role in stabilization of the cytoskeleton as it is expressed together with HSPB1 to associate with sarcomeric structures (90).

HSPB6 (HSPB20) is abundantly expressed in skeletal muscle and heart in two forms of complexs: 43-kDa dimers and 470-kDa multimers. HSPB6 has been shown to bind to itself and other sHsps (HSPB1, B5, B8) (91) and is translocated to the myofibrils in adult rat heart and skeletal muscle under ischemic conditions (92). Further evidence has demonstrated that HSPB6 binds to actin and actinin in vitro and in vivo, depending on its phosphorylation state (64,93). These findings suggest that HSPB6 may positively regulate heart function through stabilization of the cytoskeleton. Recently, it has been shown that HSPB6 overexpression resulted in significantly enhanced cardiac function by interacting with protein phosphatase-1 and enhancing Ca^{2+} cycling and sarcoplasmic reticulum Ca^{2+} load (94).

HSPB7, also known as cvHSP, is expressed in heart and skeletal muscle. Analysis of aging muscles shows a large increased level of expression of both HSPB7 and HSPB5 (95). This could reflect a cellular adapation to higher-proteotoxic stress conditions related to muscle degeneration. HSPB7 upregulation is also found in muscular dystrophy-affected diaphragm muscle, again linking high stress levels with HSPB7 induction. Furthermore, HSPB7 was recently shown to protect cells from protein aggregation, likely by facilitating cargo delivery to autophagosomes; yet unlike HSPB1, HSPB4 or HSPB5, HSPB7 could not enhance the cellular capacity to chaperone heat denatured luciferase, indicating further functional differentiation of the HSPB members

(85,96).

HpsB8 (HSP22/H11/E2IG1) is strongly expressed in striated and smooth muscles, brain, and keratinocytes. Like HSPB1, HSPB5 and HSPB8 can also be phosphorylated in vitro. In contrast to HSPB1 and HSPB5, phosphorylation only marginally affects the tertiary and quaternary structure of HSPB8. Both wild-type and phosphorylated HSPB8 exist as low-molecular mass oligomers. Unlike HSPB1 and HSPB5 where phosphorylation increase chaperone activity and reduce oligomeric size, phosphorylated HSPB8 shows a slightly larger oligomeric structure and severely lowered chaperone activity. *In vitro*, HSPB8 can form high-molecular mass hetero-oligomers with other members of HSPB family. *In vivo*, a stable and stoichiometric complex exists by binding with Bag3 and this association dramatically influences HSPB8 stability (97). In cells, HSPB8 complexes with a number of proteins; most specifically, it forms a stoichiometric complex with Bag3, a co-factor of the HSP70 (97). The Bag3/HSPB8 complex was recently shown be able to induce both translational arrest and autophagy (98,99), which may be relevant in the cellular response to irreparable protein damage.

Next to the muscle-associated HSPB members, two members (HSPB9 and HSPB10) are exclusively expressed in testis.

5. Scope of thesis

The main goal of this thesis was to get more insight in the proteolytic processes induced in cells undergoing pacing, to elucidate whether any member of the small HSPB family members may be able to protect against such AF-induced remodeling and, if so to elucidate their mechanisms of action. Hereto, we first characterized key endpoints for structural remodeling in the *in vitro* tachypaced HL-1 atrial cardiomyocyte model for AF (**Chapter 2**). Here, we investigated which myofibrillar cardiac troponin proteins are degraded in tachypaced HL-1 cardiomyocyte model and in atrial tissue of patients with AF. Furthermore, the proteases underlying troponin degradation were determined and we tested whether their inhibition leads to preservation of contractile function after AF.

In **Chapter 3**, we next investigated if a general HSP upregulation (accomplished by mild heat shock/GGA drug treatment) can protect against AF-induced structural remodeling in a tachypaced cell model. In addition, transgenic overexpression of individual HSPs (HSPA1A and HSPB1) was tested for a possible protective role in *in vitro* tachypaced HL-1 cardiomyocytes and in human AF. Here, we found that HSPB1 rather than HSPA1A might have protective effects in AF.

We therefore next compared all 10 HSPB members for their ability to protect against tachypacing-induced contractile dysfunction. Furthermore, among the protective HSPB members, the mechanism of action was determined (**Chapter 4**).

In **Chapter 5**, we decided to extend our findings from a cell model to a multicellular models system. Hereto, we developed a *Drosophila* melanogaster model for AF-induced remodeling. After validation of the model and establishing that a general HSP induction also yields AF-protective effects in this *in vivo* model, we tested various HSPB overexpressing *Drosophila* strains for their resistance towards tachypacing-induced remodeling.

Finally, in **Chapter 6**, we briefly summarized and discussed the data obtained in our experimental chapters and provide some future perspectives.

References

1. Nattel S. New ideas about atrial fibrillation 50 years on. *Nature* **2002**;415:219 - 226.

2. Brundel BJ, Ke L, Dijkhuis AJ, Qi X, Shiroshita-Takeshita A, Nattel S, Henning RH, Kampinga HH. Heat shock proteins as molecular targets for intervention in atrial fibrillation. *Cardiovasc Res.* **2008**; 78(3):422-428.

3. Fuster V, Rydén LE, Cannom DS, Crijns HJ, Curtis AB, Ellenbogen KA, *et al.* 2011 ACCF/AHA/HRS focused updates incorporated into the ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation.* **2011**;123(10):e269-367.

4. Kourliouros A, Savelieva I, Kiotsekoglou A, Jahangiri M, Camm J. Current concepts in the pathogenesis of atrial fibrillation. *Am Heart J.* **2009**;157(2):243-252.

5. Fox CS, Parise H, D'Agostino RB Sr, Lloyd-Jones DM, Vasan RS, Wang TJ, *et al.* Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring. *JAMA.* **2004**;291(23):2851-2855.

6. Saffitz JE. Connexins, conduction, and atrial fibrillation. *N Engl J Med.* **2006**;354(25):2712-2714.

7. Tsang TS, Miyasaka Y, Barnes ME, Gersh BJ. Epidemiological profile of atrial fibrillation: a contemporary perspective. *Prog Cardiovasc Dis.* **2005**;48(1):1-8.

8. Nattel S, Burstein B, Dobrev D. Atrial remodeling and atrial fibrillation: mechanisms and implications. *Circ Arrhythm Electrophysiol.* **2008**;1(1):62-73.

9. Haïssaguerre M, Jaïs P, Shah DC, Takahashi A, Hocini M, Quiniou G, *et al.* Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *N Engl J Med.* **1998**;339(10):659-666.

10. Shiroshita-Takeshita A, Brundel BJ, Nattel S. Atrial fibrillation: basic mechanisms, remodeling and triggers. *J Interv Card Electrophysiol.* **2005**;13(3):181-193.

11. Van Gelder IC, Crijns HJ, Tieleman RG, Brügemann J, De Kam PJ, Gosselink AT, *et al.* Chronic atrial fibrillation. Success of serial cardioversion therapy and safety of oral anticoagulation. *Arch Intern Med.* **1996**;156(22):2585-2592.

12. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation.* **1995**;92(7):1954-1968.

13. Nattel S, Maguy A, Le Bouter S, Yeh YH. Arrhythmogenic ion-channel remodeling in the heart: heart failure, myocardial infarction, and atrial fibrillation. *Physiol Rev.* **2007**;87(2):425-456.

14. Sun H, Gaspo R, Leblanc N, Nattel S. Cellular mechanisms of atrial contractile dysfunction caused by sustained atrial tachycardia. *Circulation*. **1998**;98(7):719-727.

15. Courtemanche M, Ramirez RJ, Nattel S. Ionic mechanisms underlying human atrial action potential properties: insights from a mathematical model. *Am J Physiol*. **1998**;275(1 Pt 2):H301-321.

16. Daoud EG, Bogun F, Goyal R, Harvey M, Man KC, Strickberger SA, Morady F. Effect of atrial fibrillation on atrial refractoriness in humans. *Circulation*. **1996**;94(7):1600-1606.

17. Qi XY, Yeh YH, Xiao L, Burstein B, Maguy A, Chartier D, *et al*. Cellular signaling underlying atrial tachycardia remodeling of L-type calcium current. *Circ Res*. **2008**;103(8):845-854.

18. Yue L, Melnyk P, Gaspo R, Wang Z, Nattel S. Molecular mechanisms underlying ionic remodeling in a dog model of atrial fibrillation. *Circ Res*. **1999**;84(7):776-784.

19. Brundel BJ, van Gelder IC, Henning RH, Tuinenburg AE, Deelman LE, Tieleman RG, *et al*. Gene expression of proteins influencing the calcium homeostasis in patients with persistent and paroxysmal atrial fibrillation. *Cardiovasc Res*. **1999**;42(2):443-454.

20. Brundel BJ, Van Gelder IC, Henning RH, Tieleman RG, Tuinenburg AE, Wietses M, *et al*. Ion channel remodeling is related to intraoperative atrial effective refractory periods in patients with paroxysmal and persistent atrial fibrillation. *Circulation*. **2001**;103(5):684-690.

21. Christ T, Boknik P, Wöhrl S, Wettwer E, Graf EM, Bosch RF, *et al*. L-type Ca2+ current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases. *Circulation*. **2004**;110(17):2651-2657.

22. Brundel BJ, Ausma J, van Gelder IC, Van der Want JJ, van Gilst WH, Crijns HJ, Henning RH. Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res*. **2002**;54(2):380-389.

23. Brundel BJ, Kampinga HH, Henning RH. Calpain inhibition prevents pacing-induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. *Cardiovasc Res*. **2004**;62(3):521-528.

24. Bosch RF, Scherer CR, Rüb N, Wöhrl S, Steinmeyer K, Haase H, *et al*. Molecular mechanisms of early electrical remodeling: transcriptional downregulation of ion channel subunits reduces I(Ca,L) and I(to) in rapid atrial pacing in rabbits. *J Am Coll Cardiol*. **2003**;41(5):858-869.

25. Yue L, Feng J, Gaspo R, Li GR, Wang Z, Nattel S. Ionic remodeling underlying action potential changes in a canine model of atrial fibrillation. *Circ Res*. **1997**;81(4):512-525.

26. Bosch RF, Zeng X, Grammer JB, Popovic K, Mewis C, Kühlkamp V. Ionic mechanisms of electrical remodeling in human atrial fibrillation. *Cardiovasc Res.* **1999**;44(1):121-131.

27. Van Wagoner DR, Pond AL, McCarthy PM, Trimmer JS, Nerbonne JM. Outward K+ current densities and Kv1.5 expression are reduced in chronic human atrial fibrillation. *Circ Res.* **1997**;80(6):772-781.

28. Goette A, Arndt M, Röcken C, Staack T, Bechtloff R, Reinhold D, *et al.* Calpains and cytokines in fibrillating human atria. *Am J Physiol Heart Circ Physiol.* **2002**;283(1):H264-272.

29. Rossow CF, Dilly KW, Santana LF. Differential calcineurin/NFATc3 activity contributes to the Ito transmural gradient in the mouse heart. *Circ Res.* **2006**;98(10):1306-1313.

30. Bukowska A, Lendeckel U, Hirte D, Wolke C, Striggow F, Röhnert P, *et al.* Activation of the calcineurin signaling pathway induces atrial hypertrophy during atrial fibrillation. *Cell Mol Life Sci.* **2006**;63(3):333-342.

31. Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, and Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation*, **1997**;96(9):3157-3163.

32. Todd DM, Fynn SP, Walden AP, Hobbs WJ, Arya S, Garratt CJ. Repetitive 4-week periods of atrial electrical remodeling promote stability of atrial fibrillation: time course of a second factor involved in the self-perpetuation of atrial fibrillation. *Circulation.* **2004**;109(11):1434-1439.

33. Sherman AJ, Klocke FJ, Decker RS, Decker ML, Kozlowski KA, Harris KR, Hedjbeli S, Yaroshenko Y, Nakamura S, Parker MA, Checchia PA, and Evans DB. Myofibrillar disruption in hypocontractile myocardium showing perfusion-contraction matches and mismatches. *Am J Physiol Heart Circ Physiol.* **2000**;278(4):1320-1334.

34. Vanoverschelde JL, Wijns W, Depré C, Essamri B, Heyndricks S, Borgers M, Bol A, and Melin J. Mechanisms of chronic regional postischemic dysfunction in humans: new insights from the study on non-infarcted collateral dependent myocardium. *Circulation.* **1993**;87(5):1513-1523.

35. Bito V, Heinzel FR, Weidemann F, Dommke C, Van Der Velden J, Verbeken E, Claus P, Bijnens B, De Scheerder I, Stienen GJM, Sutherland GR, and Sipido KR. Cellular mechanisms of contractile dysfunction in hibernating myocardium. *Circ Res.* **2004**;94(6):794-801.

36. Ausma J, Coumans WA, Duimel H, Van der Vusse GJ, Allessie M, and Borgers M. Atrial high energy phosphate content and mitochondrial enzyme activity during chronic atrial fibrillation. *Cardiovasc Res.* **2000**;47:788-796.

37. Morillo CA, Klein GJ, Jones DL, Guiraudon CM. Chronic rapid atrial pacing. Structural, functional, and electrophysiological characteristics of a new model of sustained atrial fibrillation. *Circulation.* **1995**;91(5):1588-1595.

38. Dispersyn GD, Ausma J, Thoné F, Flameng W, Vanoverschelde JL, Allessie MA, et al. Cardiomyocyte remodelling during myocardial hibernation and atrial fibrillation: prelude to apoptosis. *Cardiovasc Res.* **1999**;43(4):947-957.

39. Everett TH, Li H, Mangrum JM, McRury ID, Mitchell MA, Redick JA, *et al*. Electrical, morphological, and ultrastructural remodeling and reverse remodeling in a canine model of chronic atrial fibrillation. *Circulation.* **2000**;102(12):1454-1460.

40. Allessie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. *Cardiovasc Res.* **2002**;54(2):230-246.

41. Schotten U, Ausma J, Stellbrink C, Sabatschus I, Vogel M, Frechen D, et al. Cellular mechanisms of depressed atrial contractility in patients with chronic atrial fibrillation. *Circulation.* **2001**;103(5):691-698.

42. Frustaci A, Chimenti C, Bellocci F, Morgante E, Russo MA, Maseri A. Histological substrate of atrial biopsies in patients with lone atrial fibrillation. *Circulation.* **1997**;96(4):1180-1184.

43. Aimé-Sempé C, Folliguet T, Rücker-Martin C, Krajewska M, Krajewska S, Heimburger M, et al. Myocardial cell death in fibrillating and dilated human right atria. *J Am Coll Cardiol.* **1999**;34(5):1577-1586.

44. Hobbs WJ, Fynn S, Todd DM, Wolfson P, Galloway M, Garratt CJ. Reversal of atrial electrical remodeling after cardioversion of persistent atrial fibrillation in humans. *Circulation.* **2000**;101(10):1145-1151.

45. Söti C, Nagy E, Giricz Z, Vigh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmaco*l. **2005**;146:769 - 780.

46. Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res.* **1998**;83:117 - 132.

47. Haslbeck M, Franzmann T, Weinfurtner D, Buchner J. Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol.* **2005**;12:842 - 846.

48. Kim HJ, Hwang NR, Lee KJ. Heat shock responses for understanding diseases of protein denaturation. *Mol Cells.* **2007**;23:123 - 131.

49. Basha E, Lee GJ, Breci LA, Hausrath AC, Buan NR, Giese KC *et al.* The identity of proteins associated with a small heat shock protein during heat stress in vivo indicates that these chaperones protect a wide range of cellular functions. *J Biol Chem.* **2004**;279:7566 - 7575.

50. Jakob U, Gaestel M, Engel K, Buchner J. Small heat shock proteins are molecular chaperones. *J Biol Chem.* **1993**;268:1517 - 1520.

51. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol.* **1995**;15:505 - 516.

52. Bryantsev AL, Loktionova SA, Ilyinskaya OP, Tararak EM, Kampinga HH, Kabakow AE. Distribution, phosphorylation, and activities of Hsp25 in heat-stressed H9c2 myoblasts: a functional link to cytoprotection. *Cell Stress Chaperones.* **2002**;7:146 - 155.

53. Decker RS, Decker ML, Nakamura S, Zhao YS, Hedjbeli S, Harris KE et al. Hsc73-tubulin complex formation during low-flow ischemia in canine myocardium. *Am J Physiol Heart Circ Physiol.* **2002**;283:H1322 - H1333.

54. Van Gelder IC, Hagens VE, Bosker HA, Kingma JH, Kamp O, Kingma T, et al. A comparison of rate control and rhythm control in patients with recurrent persistent atrial fibrillation. *N Engl J Med.* **2002**;347(23):1834-1840.

55. Dobrev D, Nattel S. New antiarrhythmic drugs for treatment of atrial fibrillation. *Lancet.* **2010**;375(9721):1212-1223.

56. Plumier JC, Ross BM, Currie RW, Angelidis CE, Kazlaris H, Kollias G, and Pagoulatos GN. Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. *J Clin Invest.* **1995**;95:1854-1860.

57. Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM, and Dillmaann WH. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest.* **1995**;95:1446-1456.

58. Hayashi M, Imanaka-Yoshida K, Yoshida T, Wood M, Frearns C, Tatake RJ, and Lee JD. A crucial role of mitochondrial Hsp40 in preventing dilated cardiomyopathy. *Nature Med.* **2006**;1:128-132.

59. Brundel BJJM, Shiroshita-Takeshita A, Qi XY, Yeh YH, Chartier D, Van Gelder IC, Henning RH, Kampinga HH, and Nattel S. Induction of heat-shock response protects the heart against atrial fibrillation. *Circ Res.* **2006**;99:1394-1402.

60. Kwon JH, Kim JB, Lee KH, Kang SM, Chung N, Jang Y, and Chung JH. Protective effect of heat shock protein 27 using protein transduction domain-mediated delivery on ischemia/reperfusion heart injury. *Biochem Biophys Res Commun.* **2007**; 363:399-404.

61. Efthymiou CA, Mocanu MM, de Belleroche J, Wells DJ, Latchmann DS, and Yellon DM. Heat shock protein 27 protects the heart against myocardial infarction. *Basic Res Cardiol.* **2004**; 99:392-394.

62. Rajasekaran NS, Connell P, Christians ES, Yan LJ, Taylor RP, Orosz A, Zhang XQ, Stevenson TJ, Peshock RM, Leopold JA, Barry WH, Loscalzo J, Odelberg SJ, and Benjamin IJ. Human alpha B-crystallin mutation causes oxido-reductive stress and protein aggregation cardiomyopathy in mice. *Cell.* **2007**;130: 427-439.

63. Inagaki N, Hayashi T, Arimura T, Koga Y, Takahashi M, Shibata H, Teraoka K, Chikamori T, Yamashina A, and Kimura A. Alpha B-crystallin mutation in dilated cardiomyopathy. *Biochem Biophys Res Commun.* **2006**;342:379-386.

64. Fan GC, Yuan Q, Song G, Wang Y, Chen G, Qian J, Zhou X, Lee YJ, Ashraf M, and Kranias EG. Small heat-shock protein Hsp20 attenuates beta-agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1. *Circ Res.* **2006**;99:1233-1242.

65. Lin L, Kim SC, Wang Y, Gupta S, Davis B, Simon SI, Torre-Amione G, and Knowlton AA. HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis. *Am J Physiol Heart Circ Physiol.* 2007; 293:H2238-H2247.

66. Kupatt C, Dessy C, Hinkel R, Raake P, Daneau G, Bouzin C, Boekstegers P, and Feron O. Heat shock protein 90 transfection reduces ischemia-reperfusion-induced myocardial dysfunction via reciprocal endothelial NO synthase serine 1177 phosphorylation and threonine 495 dephosphorylation. *Arterioscler Thromb Vasc Biol.* **2004**;24:1435-1441.

67. Schafler AE, Kirmanoglou K, Balbach J, Pecher P, Hannekum A, Schumacher B. The expression of heat shock protein 60 in myocardium of patients with chronic atrial fibrillation. *Basic Res Cardiol* **2002**;97: 258 - 261.

68. Kirmanoglou K, Hannekum A, Schafler AE. Expression of mortalin in patients with chronic atrial fibrillation. *Basic Res Cardiol.* **2004**;99:404 - 408.

69. Lin KM, Lin B, Lian IY, Mestril R, Scheffler IE, Dillmann WH. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. *Circulation.* **2001**;103:1787 - 1792.

70. Yang M, Tan H, Cheng L, He M, Wie Q, Tanguay RM et al. Expression of heat shock proteins in myocardium of patients with atrial fibrillation. *Cell Stress Chaperones.* **2007**;12:142 - 150.

71. St Rammos K, Koullias GJ, Hassan MO, Argyrakis NP, Voucharas CG, Scarupa SJ *et al.* Low preoperative HSP70 atrial myocardial levels correlate significantly with high incidence of postoperative atrial fibrillation after cardiac surgery. *Cardiovasc Surg.* **2002**;10:228 - 232.

72. Mandal K, Torsney E, Poloniecki J, Camm AJ, Xu Q, Jahangiri M. Association of high intracellular, but not serum, heat shock protein 70 with postoperative atrial fibrillation. *Ann Thorac Surg.* **2005**;79:865 - 871.

73. Wakisaka O, Takahashi N, Shinohara T, Ooie T, Nakagawa M, Yonemochi H et al. Hyperthermia treatment prevents angiotensin II-mediated atrial fibrosis and fibrillation via induction of heat-shock protein 72. *J Mol Cell Cardiol.* **2007**;43:616 - 626.

74. Vos MJ, Hageman J, Carra S, Kampinga HH. Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. *Biochemistry.* **2008**;47(27):7001-7011.

75. Van Montfort R, Slingsby C, Vierling E. Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. *Adv Protein Chem.* **2001**;59:105-156.

76. Mogk A, Schlieker C, Friedrich KL, Schönfeld HJ, Vierling E, Bukau B. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J Biol Chem.* **2003**;278(33):31033-31042.

77. Veinger L, Diamant S, Buchner J, Goloubinoff P. The small heat-shock protein IbpB from Escherichia coli stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J Biol Chem.* **1998**;273(18):11032-11037.

78. Landry J, Huot J. Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem Cell Biol.* **1995**;73(9-10):703-707.

79. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol.* **1995**;15(1):505-516.

80. Kamradt MC, Chen F, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem.* **2001**;276(19):16059-16063.

81. Suzuki A, Sugiyama Y, Hayashi Y, Nyu-i N, Yoshida M, Nonaka I, *et al.* MKBP, a novel member of the small heat shock protein family, binds and activates the myotonic dystrophy protein kinase. *J Cell Biol.* **1998**;140(5):1113-1124.

82. Sugiyama Y, Suzuki A, Kishikawa M, Akutsu R, Hirose T, Waye MM, *et al*. Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. *J Biol Chem*. **2000**;275(2):1095-1104.

83. Yoshida K, Aki T, Harada K, Shama KM, Kamoda Y, Suzuki A, Ohno S. Translocation of HSP27 and MKBP in ischemic heart. *Cell Struct Funct*. **1999**;24(4):181-185.

84. Feil IK, Malfois M, Hendle J, van Der Zandt H, Svergun DI. A novel quaternary structure of the dimeric alpha-crystallin domain with chaperone-like activity. *J Biol Chem*. **2001**;276(15):12024-12029.

85. Vos MJ, Zijlstra MP, Carra S, Sibon OC, Kampinga HH. Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy*. **2011**;7(1):101-103.

86. Lutsch G, Vetter R, Offhauss U, Wieske M, Gröne HJ, Klemenz R, *et al*. Abundance and location of the small heat shock proteins HSP25 and alphaB-crystallin in rat and human heart. *Circulation*. **1997**;96(10):3466-3476.

87. Bullard B, Ferguson C, Minajeva A, Leake MC, Gautel M, Labeit D, *et al*. Association of the chaperone alphaB-crystallin with titin in heart muscle. *J Biol Chem*. **2004**;279(9):7917-7924.

88. Martin JL, Mestril R, Hilal-Dandan R, Brunton LL, Dillmann WH. Small heat shock proteins and protection against ischemic injury in cardiac myocytes. *Circulation*. **1997**;96(12):4343-8.

89. Rajasekaran NS, Connell P, Christians ES, Yan LJ, Taylor RP, Orosz A, *et al*. Human alpha B-crystallin mutation causes oxido-reductive stress and protein aggregation cardiomyopathy in mice. *Cell*. **2007**;130(3):427-439.

90. Vicart P, Caron A, Guicheney P, Li Z, Prévost MC, Faure A, *et al*. A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat Genet*. **1998**;20(1):92-95.

91. Pipkin W, Johnson JA, Creazzo TL, Burch J, Komalavilas P, Brophy C. Localization, macromolecular associations, and function of the small heat shock-related protein HSP20 in rat heart. *Circulation*. **2003** ;107(3):469-476.

92. Golenhofen N, Perng MD, Quinlan RA, Drenckhahn D. Comparison of the small heat shock proteins alphaB-crystallin, MKBP, HSP25, HSP20, and cvHSP in heart and skeletal muscle. *Histochem Cell Biol*. **2004**;122(5):415-425.

93. Dreiza CM, Brophy CM, Komalavilas P, Furnish EJ, Joshi L, Pallero MA, et al. Transducible heat shock protein 20 (HSP20) phosphopeptide alters cytoskeletal dynamics. *FASEB J*. **2005**;19(2):261-263.

94. Qian J, Vafiadaki E, Florea SM, Singh VP, Song W, Kung Lam C, et al. Small Heat Shock Protein 20 Interacts With Protein Phosphatase-1 and Enhances Sarcoplasmic Reticulum Calcium Cycling. *Circ Res.* **2011** Apr 14.

95. Doran P, Gannon J, O'Connell K, Ohlendieck K. Aging skeletal muscle shows a drastic increase in the small heat shock proteins alphaB-crystallin/HspB5 and cvHsp/HspB7. *Eur J Cell Biol.* **2007**;86(10):629-640.

96. Vos MJ, Zijlstra MP, Kanon B, van Waarde-Verhagen MA, Brunt ER, Oosterveld-Hut HM, et al. HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones. *Hum Mol Genet.* **2010**;19(23):4677-4693.

97. Carra S, Seguin SJ, Lambert H, Landry J. HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem.* **2008**;283(3):1437-1444.

98. Carra S. The stress-inducible HspB8-Bag3 complex induces the eIF2alpha kinase pathway: implications for protein quality control and viral factory degradation? *Autophagy.* **2009**;5(3):428-429.

99. Carra S, Boncoraglio A, Kanon B, Brunsting JF, Minoia M, Rana A, et al. Identification of the Drosophila ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J Biol Chem.* **2010**;285(48):37811-37822.

CHAPTER 2

Calpain mediates cardiac troponin degradation and contractile dysfunction in Atrial Fibrillation

Lei Ke [a], XiaoYan Qi [b], Anne-Jan Dijkhuis [a], Denis Chartier [b], Stanley Nattel [b], Robert H. Henning [c], Harm H. Kampinga [a], Bianca J.J.M. Brundel [a,c]

[a] Department of Radiation and Stress Cell Biology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands

[b] Department of Medicine and Research Center, Montreal Heart Institute and University of Montreal, Quebec, Canada

[c] Department of Clinical Pharmacology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands.

J Mol Cell Cardiol. 2008 Nov;45(5):685-693.

Abstract

The self-perpetuation of atrial fibrillation (AF) is associated with atrial remodeling, including the degradation of the myofibril structure (myolysis). Myolysis is related to AF-induced activation of cysteine proteases and underlies loss of contractile function. In this study, we investigated which proteases are involved in the degradation of myofibrillar proteins during AF and whether their inhibition leads to preservation of contractile function after AF. In tachypaced HL-1 cardiomyocytes and atrial tissue from AF and control patients, degradation of myofibrillar proteins troponin (cTn) T, I, C, human cTnT and actin was investigated by Western blotting, and contractile function was analyzed by cell-shortening measurements. The role of major proteases was determined by applying specific inhibitors. Tachypacing of HL-1 cardiomyocytes induced a gradual and significant degradation of cTns but not actin, and caused contractile dysfunction. Both were prevented by inhibition of calpain but not by inhibition of caspases or the proteasome. In patients with persistent AF, a significant degradation of cTnT, cTnI and cTnC was found compared to sinus rhythm or paroxysmal AF, which correlated significantly with both calpain activity and the amount of myolysis. Additionally, by utilizing tachypaced human cTnT-transfected HL-1 cardiomyocytes, we directly showed that the degradation of human cTnT was mediated by calpain and not by caspases or proteasome. Our results suggest that calpain inhibition may therefore represent a key target in combating AF-related structural and functional remodeling.

Introduction

Atrial Fibrillation (AF), the most common clinical tachyarrhythmia and an important contributor to cardiovascular morbidity and mortality (1), tends to become more persistent over time (2,3). The mechanisms underlying self-perpetuation are linked to rapid changes in electrical and contractile functions of cardiomyocytes. When the arrhythmia persists, AF induces changes at the structural level, predominantly myolysis (4,5). Myolysis is characterized by loss of myofibril structure, leading to contractile dysfunction (6,7) and AF progression (4,5). AF is accompanied by reduced expression levels of all three subunits of the myofibrillar troponin complex, cTnT, cTnI and cTnC (8,9), which is the main regulatory component of cardiac contraction in response to a rise of intracellular Ca^{2+} concentration (10). Therefore, identification of therapeutic approaches and mechanisms that stabilize the troponin complex during AF are important for the attenuation of AF-induced atrial remodeling.

We previously showed increased activation of the Ca^{2+}-dependent neutral protease calpain in both human AF and a cell model for tachypacing-induced remodeling (11,12). Calpains degrade cytoskeletal and contractile proteins (13), including cardiac troponins (13,14). We therefore hypothesized that AF causes calpain-induced troponin degradation, resulting in contractile and structural remodeling. Here, we exploit tachypaced HL-1 cardiomyocytes to study the involvement of several proteases in the degradation of the myofibrillar proteins cTnT, cTnI, cTnC and actin. We also explore whether prevention of troponin degradation conserves contractile function. Additionally, we collected samples from patients with paroxysmal (PAF) and persistent (PeAF) AF to analyze alterations in myofibrillar protein expression. To directly test whether human cardiac troponins are targets for calpain-induced degradation, HL-1 cardiomyocytes were transfected with human cTnT and subjected to tachypacing in combination with various protease inhibitors. We obtained definitive evidence that calpain mediates cardiac troponin degradation and contractile dysfunction in *in vitro* and human tissue models for AF.

Materials and Methods

HL-1 cardiomyocyte culture, tachypacing and major protease inhibition

HL-1 atrial cardiomyocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, USA) (15). The cardiomyocytes were maintained in Complete Claycomb Medium (JRH, UK) supplemented with 100μM norepinephrine (Sigma, The Netherlands) dissolved in 0.3mM L-ascorbic acid (Sigma), 4mM L-glutamine (Gibco, The Netherlands) and 10% FBS (Life Technologies, Gaithersburg, MD). They were cultured in flasks coated with 12.5μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma), in a 5% CO_2 atmosphere at 37°C.

The spontaneous rate of HL-1 cardiomyocytes is ~0.5-1Hz. To induce tachycardia, HL-1 cardiomyocytes ($\geq 1\times10^6$ cells) were cultured on 4-well rectangular dishes (Nuclon, The Netherlands), placed into C-Dish100™-Culture Dishes (IonOptix Corporation, MA) and subjected to electrical field stimulation (3Hz) via the C-Pace100™-Culture Pacer (IonOptix Corporation, The Netherlands) as described previously (12,16,17). We used 1Hz pacing as a control.

Calpain inhibitor PD150606, pan-caspase inhibitor Z-VAD-FMK and proteasome inhibitor MG132 were purchased from Calbiochem (The Netherlands). The pan-caspase inhibitor Z-VAD-FMK inhibits all caspases. HL-1 cardiomyocytes were treated with PD150606 (20μM), Z-VAD-FMK (50μM) or MG132 (10μM) two hours prior to tachypacing to achieve complete inhibition as determined by individual enzymatic assays.

Construct, transfection and detection of fusion protein

To study degradation of human cTnT, HL-1 cardiomyocytes were transiently transfected with the plasmid pcDNA5/FRT/TO (Invitrogen, USA) driving the expression of full length human cTnT. The primers CGGGATCCACCATGTCTGACATAGAAGAG GTGGTGGAAG (forward) and AAGAATGCGGCCGCTTTCCAGCGCCCGGT

GACTTTAG (reverse) were used to amplify human cTnT with PCR from a human heart cDNA library (Stratagene, USA). To obtain V5-pcDNA5/FRT/TO vector, the 14 amino acid V5 epitope was cloned in the NotI and ApaI sites of pcDNA5/FRT/TO. In addition, cTnT was subsequently cloned in the BamHI and NotI sites of V5-pcDNA5/FRT/TO vector to generate pV5-C-hucTnT. Lipofectamine (Life technologies, The Netherlands) was used to transiently transfect HL-1 cardiomyocytes according to manufacturer instructions. Expression of V5-C-hucTnT recombinant protein in cardiomyocytes was determined by Western blotting with mouse monoclonal Anti-V5-Antibody (R960-25, Invitrogen, USA).

Measurement of calpain, caspase and proteasome activity

Calpain

Calpain enzymatic activity was determined with the use of a calpain activity assay kit (K240-100, BioVision, USA). Briefly, 1×10^6 cardiomyocytes were harvested and counted upon trypsinization. After cell suspension in 100µl Extraction Buffer, samples were incubated on ice for 20 minutes. The cell lysate was centrifuged for 1 minute (10,000g) and the supernatant was transferred to a fresh tube and put on ice. After protein-concentration determination (RC DC Protein Assay, Bio-Rad, The Netherlands), each sample was diluted into Extraction Buffer (50µg protein/85µl), followed by the addition of 10µl of 10× Reaction Buffer and 5µl Calpain Substrate to each assay-sample. Calpain-activity was measured by fluorometry (400-nm excitation; 505-nm emission) immediately after 1-hour incubation of samples in the dark at 37℃.

The calpain activity measurement in human tissue was performed as described previously (11). Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (AMC, Sigma, The Netherlands) was used as substrate. Protein extract (25µg) was added to 20µM AMC in 300µl Tris-buffered saline. AMC release was measured by fluorometry (360-nm excitation; 430-nm emission, Spectrometer LS50B, PerkinElmer, The Netherlands) after 30-min

incubation at room temperature.

Caspase

Caspase activity was measured using a caspase fluorometric assay kit (K105-100, BioVision, USA). Briefly, 1×10^6 cardiomyocytes were harvested and counted upon trypsinization. Cells were suspended in 50μl of chilled Cell Lysis Buffer and then were incubated on ice for 10 minutes. 50μl of 2×Reaction Buffer and 5μl of 1mM DEVD-AFC substrate (50μM final concentration) was added to each sample, followed by the incubation at 37℃ for 2 hours. Finally, all samples were read in a fluorometer with 400-nm excitation and 505-nm emission.

Proteasome

To determine involvement of the proteasome, HL-1 cardiomyocytes were transfected with a construct encoding Ubiquitin-R-GFP fusion protein (18). Ubiquitin-R-GFP converts stable GFP into a substrate vulnerable to ubiquitin–proteasome-dependent proteolysis. GFP turnover is then mediated by proteasome activity, and increased amounts of GFP indicate proteasome inhibition. To determine proteasome activity, cardiomyocytes were treated with or without the proteasome inhibitor MG132 two hours before and during tachypacing. After 24 hours of tachypacing, flow cytometry was used to analyze the difference in the percentage of GFP positive cardiomyocytes between MG132-treated and MG132-non-treated cells. Cardiomyocytes paced at 1Hz with or without MG132 treatment served as controls.

Contractility assessment by cell-shortening measurement and live imaging of Ca^{2+} transients

Cell-shortening (CS) was measured as described previously (16,19). Briefly, CS (maximum minus minimum cell-length) was measured with a video edge detector

(Crescent Electronics) coupled to a charge-coupled device camera. The contraction signal was digitized at 200 Hz (TL-1 A/D Convert, Axon). Edge-detection cursors were positioned at both cardiomyocyte-ends to measure whole-cell shortening. CS was measured relative to diastolic cell-length based on the average of 10 consecutive beats. Ca^{2+} transients were imaged by Solamere-Nipkow-Confocal-Live-Cell-Imaging system (based on a Leica DM IRE2 Inverted microscope). 2μM of the Ca^{2+}-sensitive Fluo-4-AM dye (Invitrogen) was loaded into HL-1 cardiomyocytes by 45-min incubation, followed by 3 times washing with PBS. Ca^{2+} loaded cardiomyocytes were excited by 488 nm and emitted at 500-550 nm and visually recorded with a 40x-objective.

Patients

Prior to surgery, one investigator assessed patient characteristics (Table 1) and classified arrhythmia history according to Gallagher and Camm (20). Persistent (n=17) and paroxysmal (n=14) groups contained patientswith lone AF or AFwith underlying mitral valve disease (MVD). All patients were euthyroid and had normal left-ventricular function. Coumarin therapy was interrupted 3 days before surgery and class I and III anti-arrhythmic drugs were discontinued for at least 5 half-lives. Right and/or left atrial appendages (RAAs and LAAs respectively), were obtained from all patients, except for the control patients undergoing CABG from whom only the RAA was gathered prior to cardiopulmonary bypass (Table 1). After excision, atrial appendages were immediately snap-frozen in liquid nitrogen and stored at 85 °C. The investigation conforms to the principles of the Declaration of Helsinki. The Institutional Review Board approved the study and patients gave written informed consent. Samples from the same patients were used in a previous study (17). The amount of myolysis was quantified as described previously (17). An atrial myocyte was defined as myolytic when >10% of the myocyte surface was free from myosin staining. Five randomly chosen fields or more of a total of 250 - 500 myocytes were examined by three independent observers blinded for the experimental groups. Mean scores of the observers were used.

Table 1 Baseline demographic and clinical characteristics of patients with paroxysmal AF (PAF), persistent AF (PeAF) and control patients in sinus rhythm

	SR	PAF	PeAF
N	13	14	17
RAA (*n*)	11	12	16
LAA (*n*)	5	12	16
Age	61±4	50±3	53±3
Duration of AF (median, range (months))	–	–	11.6 (0.1–56)
Duration SR before surgery (median, range (days))	–	10 (0.5–210)	–
Duration of last episode AF (median, range (h))	–	12 (0.2–24)	–
AF/day (median, range (%))	–	2 (0.2–70)	–
Underlying heart disease (*n*) and/or surgical procedure			
Coronary artery disease/CABG	8*	0	0
Lone AF/Maze	0	8	9
MVD/MV replacement/repair	5	6	8
New York Heart Association for exercise tolerance			
Class I	10	6	5
Class II	3	5	8
Class III	0	3	4
Echocardiography			
Left atrial diameter (parasternal)	42±3	42±4	48±4
Left ventricular end-diastolic diameter (mm)	50±4	52±3	52±3
Left ventricular end-systolic diameter (mm)	34±4	38±3	34±5
Medication (*n*)			
Ace-inhibitors	4	5	7
Digitalis	0	1	7
Verapamil	4	3	4
Beta-blocker	6	3	4

Values are presented as mean value±SEM or number of patients. CABG: Coronary Artery Bypass Grafting; Maze: atrial arrhythmia surgery; MVD: mitral valve disease. *$p<0.05$.

Protein-extraction and Western blot analysis

Western blot analysis was performed as described previously (17,21). Equal amount of protein in SDS-PAGE sample buffer was sonicated before separation on 10% PAA-SDS gels. After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against GAPDH (Affinity Reagents,

USA), cTnT (RDI-4T19-1A11), cTnI (RDI-TRK4T21-MF4), cTnC (RDI-4T27-1A2), cardiac actin (RDI-PRO61075), (all from RDI Division of Fitzgerald Industries Intl, USA). Horseradish peroxidase-conjugated anti-mouse (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry. The amount of protein chosen was in the linear immunoreactive signal range and expressed relative to GAPDH.

Statistical analysis

Results are expressed as mean ± SEM. All Western-blot procedures were performed in at least duplicate series. ANOVA was used for multiple-group comparisons. Correlation of human data was determined using the Spearman correlation test, which is a non-parametric measure of correlation for small group numbers. All p values were two-sided. $p< 0.05$ was considered statistically significant. SPSS version 8.0 was used for statistical evaluation.

Results

Tachypacing of HL-1 cardiomyocytes induces troponin degradation and activation of calpain and caspases

To determine the putative consequences of tachypacing on myofibril degradation, we measured expression levels of troponins and actin in HL-1 cardiomyocytes. Exposure of HL-1 cardiomyocytes to tachypacing (3Hz) for up to 24 hours resulted in a gradual loss of cTnT, cTnI and cTnC, compared to 1Hz pacing. Actin expression-levels were unaffected by tachypacing (Figure 1A). As observed previously (11,12), 24 hours of tachypacing of HL-1 cardiomyocytes resulted in significant induction of calpain activity, which was inhibited by pre-treatment with the specific calpain inhibitor PD150606 (20μM, Figure 1B). Since caspases and the ubiquitin-proteasome systems are involved in degradation of cardiac proteins and found to promote cardiac dysfunction in ventricular cardiomyocytes (22-24), the effect of tachypacing on caspase activity and on the proteasome was also studied. Tachypacing indeed induced caspases activation, which was inhibited by treating cardiomyocytes prior to and during tachypacing with the pan-caspase inhibitor Z-VAD-FMK (50μM, Figure 1C). To assess the contribution of the proteasome to tachypacing-induced cardiac troponin degradation, cardiomyocytes were transiently transfected with Ubiquitin-R-GFP fusion reporter (18) with or without the proteasome inhibitor MG132 (10μM). FACS analysis revealed that the percentage of GFP positive cardiomyocytes was unchanged after 24 hours of tachypacing (Figure 1D), compared to 1Hz. Adding MG132 to both 1Hz and 3Hz paced cardiomyocytes caused accumulation of GFP, demonstrating that MG132 effectively inhibited the proteasome activity (Figure 1D).

Calpain-mediated degradation of cardiac troponins

To more conclusively determine whether calpain directly degrades cardiac troponins in

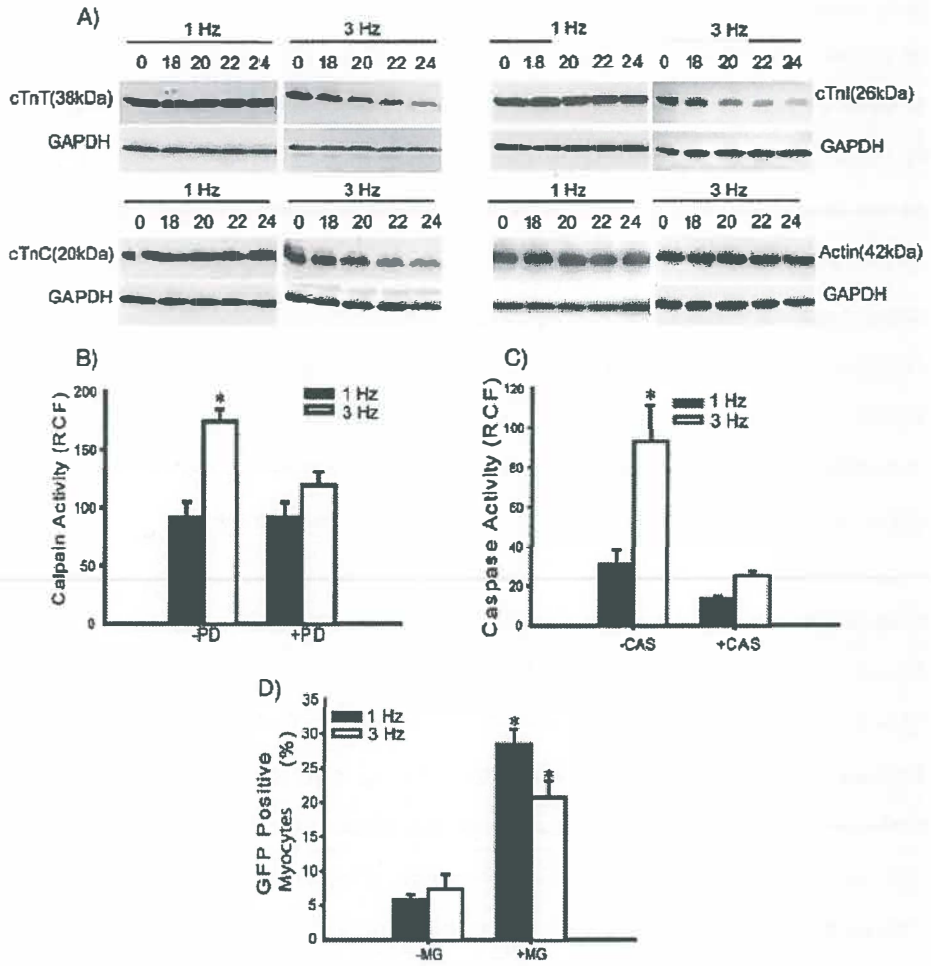


Figure 1 Tachypacing-induced degradation of cardiac troponins and activation of calpain and caspases. **(A)** Representative Western blots show that tachypacing (3Hz) decreases cTnT, cTnI and cTnC levels, compared to 1Hz control cardiomyocytes. No alteration in the expression of actin was observed. **(B)** HL-1 cardiomyocytes were paced at 1Hz or 3Hz in the absence of PD150606 (-PD) or in the presence of PD150606 (+PD). Calpain activity was measured by monitoring fluorescence after specific cleavage of the test substrate. N=4 per group. *p<0.05 (3Hz-PD vs 1Hz-PD). **(C)** HL-1 cardiomyocytes were 3Hz tachypaced or 1Hz paced in the absence (-CAS) or in the presence (+CAS) of pan-caspase inhibitor Z-VAD-FMK. Caspase activity was measured by monitoring fluorescence after specific cleavage of the test substrate. N=4 per group. *=p<0.05 (3Hz-CAS vs 1Hz–CAS). **(D)** Proteasome activity was measured in 3Hz and 1Hz paced HL-1 cardiomyocytes pre-incubated either without (-MG) or with (+MG) the proteasome inhibitor MG132. Bar graphs represent the percentage of GFP positive HL-1 cardiomyocytes. *=p<0.05 (+MG vs –MG).

tachypaced HL-1 cardiomyocytes, cardiomyocytes were treated with the calpain inhibitor PD150606 (20μM) prior to and during tachypacing. The mean amount of cardiac troponin expression is shown relative to GAPDH and plotted versus pacing duration. PD150606 prevented tachypacing-induced degradation of cTnT, cTnI and cTnC (Figure 2A-C). Incubation of HL-1 cardiomyocytes with the pan-caspase inhibitor Z-VAD-FMK (50μM) did not attenuate cTnT, cTnI or cTnC degradation (Figure 2D), suggesting no involvement of caspases in tachypacing-induced troponin degradation. In addition, degradation of cTnT, cTnI and cTnC was unaffected in tachypaced cardiomyocytes incubated with MG132 (10μM, Figure 2E), indicating no involvement of the proteasome. In summary, the findings suggest that calpain, and not caspases or the proteasome, is the key component in tachypacing-induced degradation of cardiac troponins.

Calpain-inhibition prevents tachypacing-induced contractile dysfunction

To study if calpain-mediated cardiac troponin degradation is directly linked to contractile dysfunction, the effect of calpain-inhibition on the degree of cell shortening (CS) of HL-1 cardiomyocytes was assessed. While tachypacing markedly reduced CS of HL-1 cardiomyocytes compared to 1Hz paced cardiomyocytes, the calpain inhibitior PD150606 (20μM) completely prevented CS-depression (Figure 3). Pan-caspases and the proteasome inhibition did not attenuate tachypacing-induced contractile dysfunction in HL-1 cardiomyoctyes (data not shown). The effect of PD150606 on tachypacing-induced changes in Ca^{2+} transients was studied with live imaging. Tachypacing for 4 h induced loss of rhythmic fluorescence-signal, indicating reductions in Ca^{2+} transients, but PD150606 conserved Ca^{2+} transients (supplemental information movies 1-3). These observations demonstrate that calpain inhibition attenuates both tachypacing-induced cardiac troponin degradation and contractile dysfunction in HL-1 cardiomyocytes.

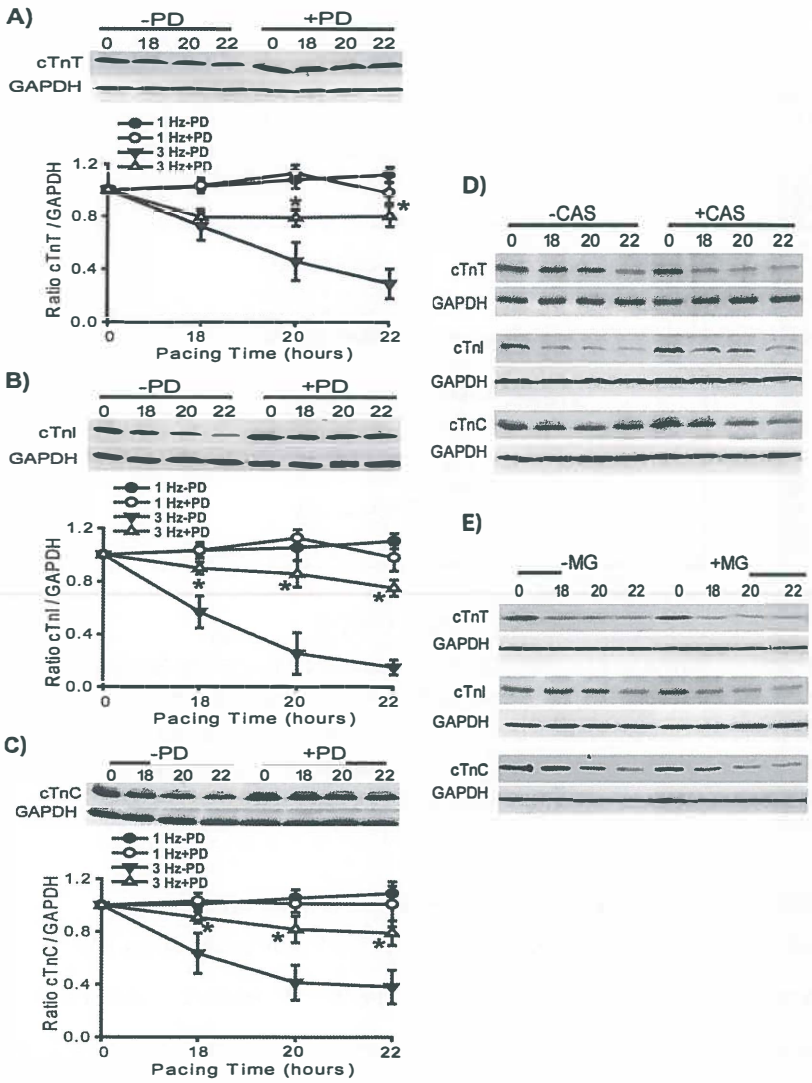


Figure 2 Calpain-mediated degradation of cardiac troponins. Top panels show representative immunoblots of cTnT **(A)**, cTnI **(B)**, and cTnC **(C)** in tachypaced (3Hz) HL-1 cardiomyocytes, either in the absence (-PD) or the presence of (+PD) calpain inhibitor PD150606. Bottom, the mean expression levels of cTnT, cTnI and cTnC are shown. N≥4 for each data point. *p<0.05 (3Hz+PD vs 3Hz-PD). Control groups were paced at 1Hz, either in the absence (-PD) or the presence of (+PD) calpain inhibitor PD150606. **(D)** Representative immunoblots of cTnT, cTnI and cTnC in tachypaced (3Hz) HL-1 cardiomyocytes, either in the absence of (-CAS) or the presence of pan-caspase inhibitor (+CAS). **(E)** Representative immunoblots of cTnT, cTnI and cTnC in tachypaced (3Hz) HL-1 cardiomyocytes, either in the absence of (-MG) or the presence of MG132 (+MG).

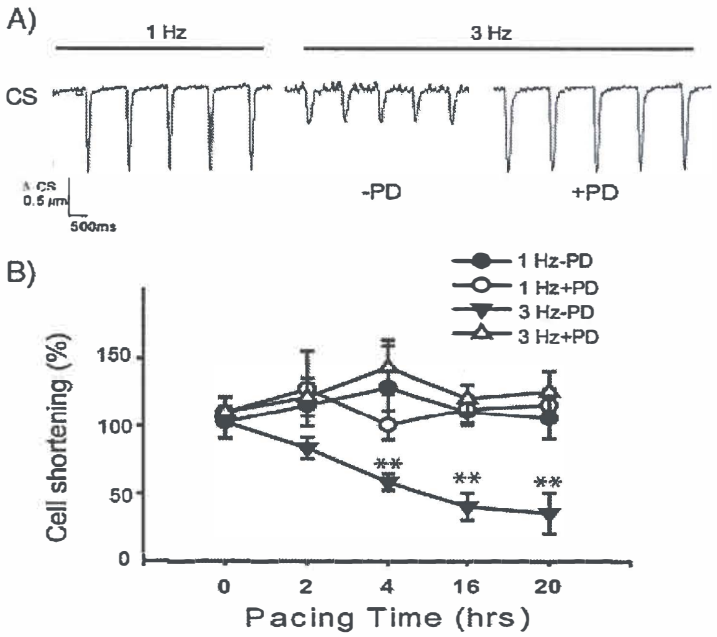


Figure 3 Calpain inhibition prevents tachypacing-induced contractile dysfunction. **(A)** Original recordings of cell shortening (CS) in one cell each from groups indicated. **(B)** CS was measured in tachypaced (3Hz) and 1Hz paced HL-1 cardiomyocytes either in the absence (-PD) or the presence of (+PD) calpain inhibitor PD150606. Tachypacing significantly resulted in the reduction of CS, while the calpain inhibitor PD150606 prevents CS reduction. N=8 to 12 cells/data point. $^{**}p<0.01$ vs 0 hour

Degradation of cardiac troponins in patients with atrial fibrillation

To extend the findings on calpain-mediated degradation of cardiac troponins in the cell model in relation to high levels of myolysis in PeAF (11), cardiac troponin levels were determined in atrial tissue from PeAF, PAF and sinus rhythm (SR) control patients. Results are shown in Figure 4, with patients lacking known atrial disease in filled circles and mitral valve disease patients in open symbols. PeAF patients showed a significant reduction in cTnT levels (38 kDa), compared to SR patients (Figure 4A). Moreover, after longer exposure of the blot, a 25 kDa cleavage product was observed in PeAF, but not in SR and PAF (Figure 4B). The expression levels of cTnI (Figure 4C) and cTnC (Figure 4E) were significantly reduced in patients with PeAF compared to patients with either SR or

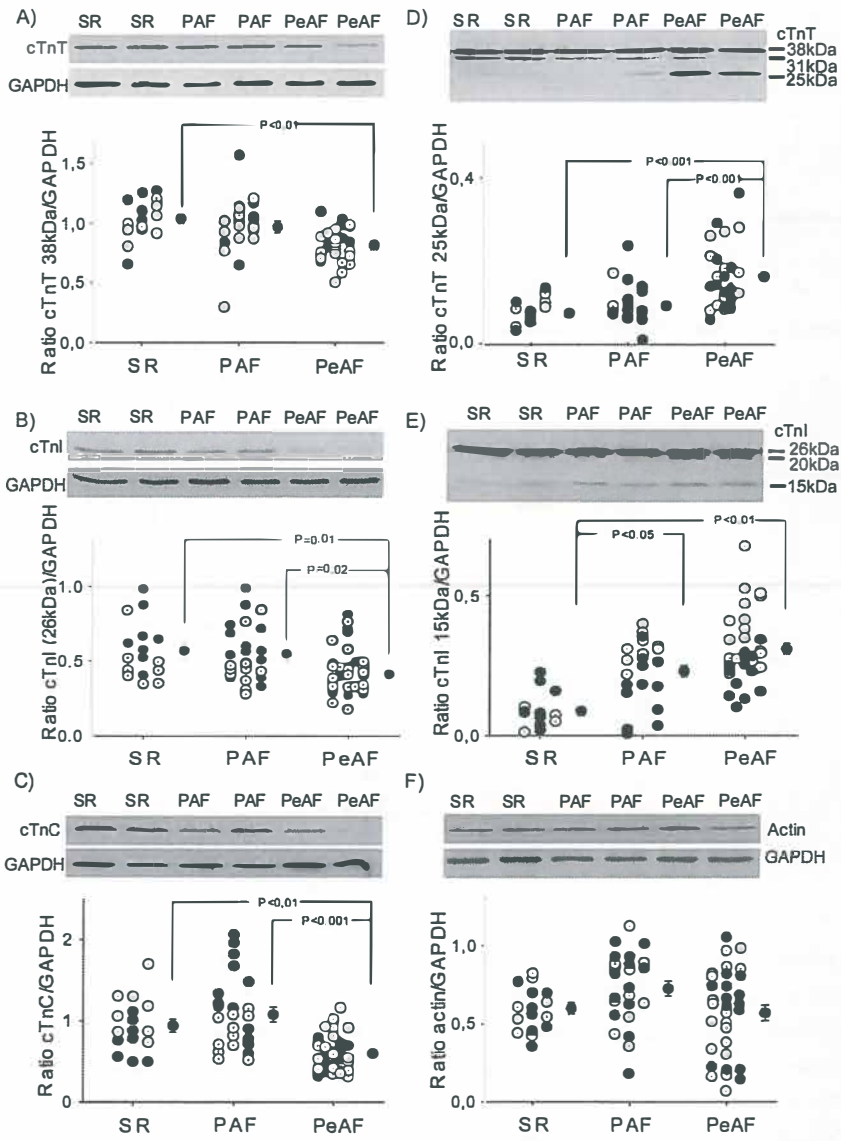


Figure 4 Patients with persistent AF (PeAF) show degradation of troponins. Representative immunoblots showing the decreased expression of full length cTnT **(A)**, cTnI **(B)**, cTnC **(C)** in atrial tissue of patients with persistent AF (PeAF) compared to paroxysmal AF (PAF) and controls in sinus rhythm (SR). In overexposed blots, a significant increase in the degradation product for cTnT (25kDa) **(D)** and cTnI (15kDa) **(E)** is observed in PeAF compared with PAF and SR. All groups show similar expression of actin (F). (●) represents lone AF patients or SR patients undergoing CABG, (□) represents patients with mitral valve disease.

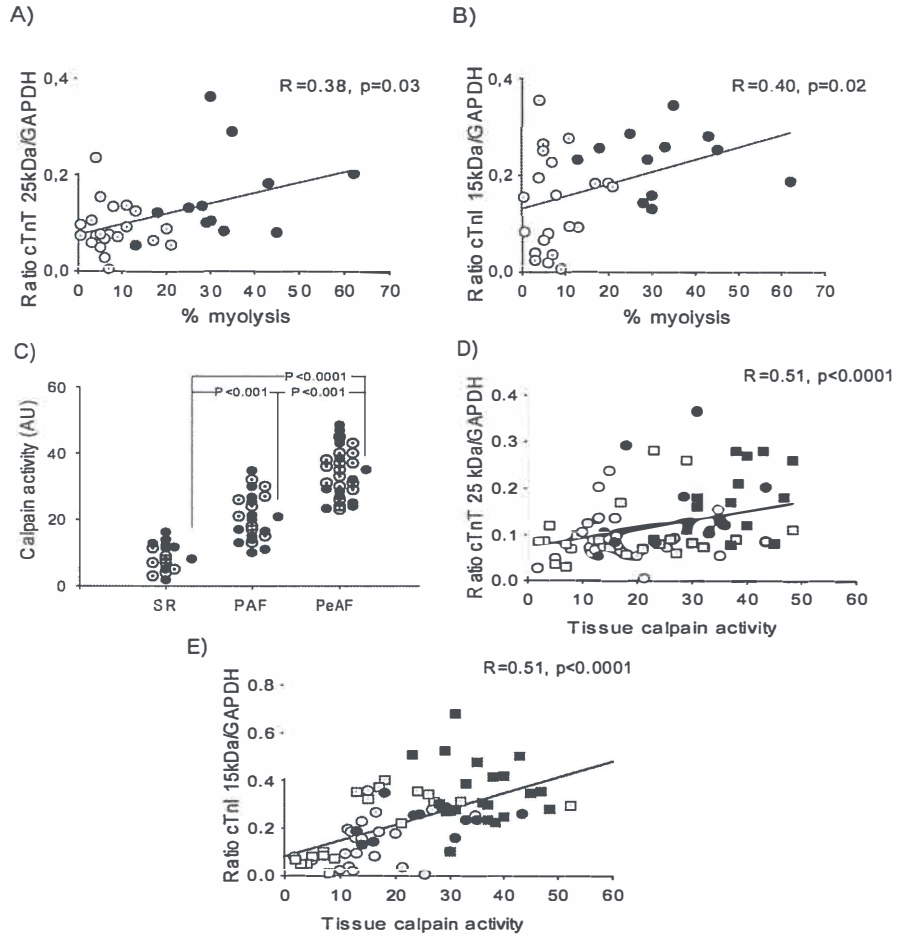


Figure 5 Myolysis and calpain-activity correlate with the amount of troponin degradation products in AF patients. **(A)** There is a significant correlation between the degree of myolysis and the amount of human 25kDa cTnT degradation-product (R=0.38, p=0.03). **(B)** The significant correlation between the degree of myolysis and the amount of human 15kDa cTnI degradation-product (R=0.40, p=0.02). **(C)** Significant induction of calpain was found both in PAF and PeAF patients, compared to patients with SR. **(D)** The significant correlation between calpain activity and the amount of human 25kDa cTnT degradation-product (R=0.51, p<0.0001). **(E)** The significant correlation between calpain activity and the amount of human 15kDa cTnI degradation-product (R=0.51, p<0.0001). (●) represents lone persistent AF patients, (□) represents patients with lone paroxysmal AF, (○) represents SR patients undergoing CABG, (□) represents SR patients with mitral valve disease, (⊡) represents paroxysmal AF patients with mitral valve disease, (■) represents persistent AF patients with mitral valve disease.

PAF. For cTnI, a 15 kDa degradation fragment was detected in PeAF patients (Figure 4D). For cTnC, no degradation fragments were detected. Consistent with our *in vitro* data, no alterations in the expression levels of actin were observed (Figure 4F).

Previously, we reported ultrastructural changes in atrial tissue of a part of this patient population (11). In patients with lone persistent AF, a substantial fraction of cells was myolytic (30.0±14.5%), whereas in tissue of patients with lone paroxysmal AF myolysis was low (6.9±6.1%) and similar to control patients (5.5±3.6%). In the lone AF patients in the present study, we found a significant correlation between the extent of myolysis and the amounts of 25 kDa cTnT and 15 kDa cTnI degradation products (Figure 5A, B, respectively). This observation indicates that myolysis is associated with troponin degradation product accumulation.

To study whether a relation exists between calpain activity and human cTnT and cTnI degradation, calpain activity was measured in tissue samples from AF patients and SR controls. A significant induction of calpain activity was observed in PAF (2-fold increase, $p<0.001$) and PeAF (3.5-fold increase, $p<0.0001$) vs SR controls (Figure 5C). Significant correlations were found between calpain activity and cTnT 25 kDa and cTnI 15 kDa degradation products (Figure 5D, E), i.e. high calpain activity was associated with large amounts of troponin degradation products. Together, the human findings are in accordance with our observations in tachypaced HL-1 cardiomyocytes and suggest a causative relationship between AF-induced calpain activation and troponin degradation.

Tachypacing-induced degradation of human cTnT in HL-1 cardiomyocytes

To establish whether the 25 kDa human cTnT fragment results from cleavage by calpains, HL-1 cardiomyocytes were transfected with a tagged human cTnT (pV5-C-hucTnT) and subjected to tachypacing. With the V5 antibody, the 25 kDa degradation product of V5-C-hucTnT fusion protein was detected in tachypaced cardiomyocytes (Figure 6), while pre-treatment with the calpain inhibitor PD150606 (20 μM) completely abolished

its appearance (Figure 6). No attenuation of the tachypacing-induced 25 kDa V5-C-hucTnT degradation product was observed in cardiomyocytes pretreated with pan-caspases or proteasome inhibitor (Figure 6). These findings confirm that calpain activation mediates the cleavage of human cTnT into a 25 kDa fragment, as observed in patients with PeAF.

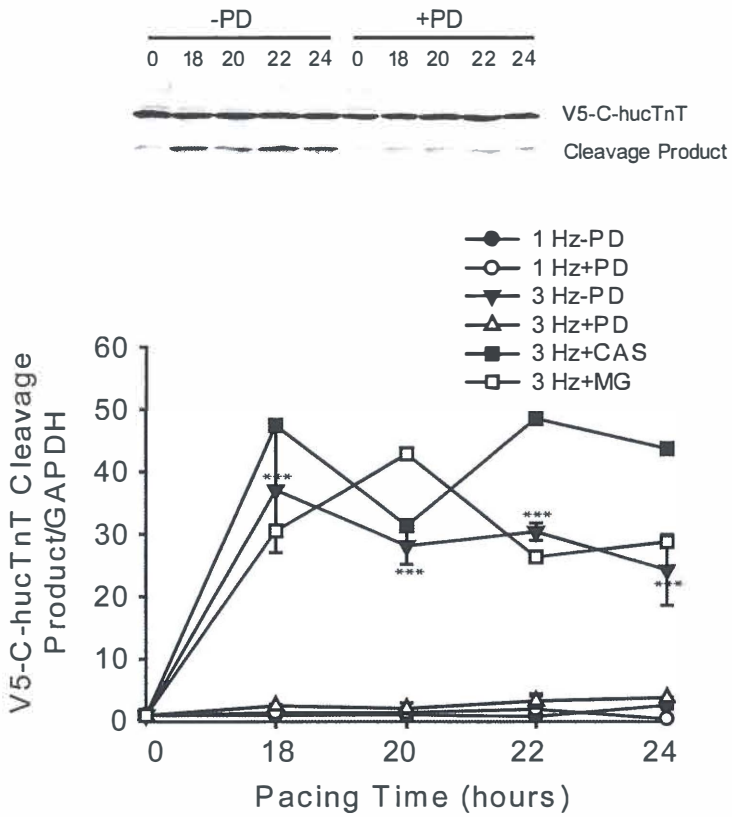


Figure 6 Tachypacing induces degradation of human cTnT in HL-1 cardiomyocytes. V5-C-hucTnT transfected HL-1 cardiomyocytes were tachypaced (3Hz) or paced at 1Hz in the absence of (-PD) or in the presence (+PD) of the calpain inhibitor PD150606, pan-caspase inhibitor Z-VAD-FMK (+CAS) and the proteasome inhibitor MG132 (+MG). Top shows typical Western blot demonstrating the appearance of a cTnT degradation product of 25kDa by tachypacing, which was abolished by PD150606. Bottom, quantification of V5-C-hucTnT cleavage products. ***p<0.001 (3Hz+PD vs 3Hz-PD).

Discussion

In this study, we show that *in vitro* tachypacing of HL-1 atrial cardiomyocytes results in the gradual degradation of cTnT, cTnI and cTnC, which is exlusively mediated by calpain activation. In addition, contractile dysfunction of tachypaced cardiomyocytes was prevented by calpain inhibition. In atrial tissue from patients with PeAF, a significant degradation of cTnT, cTnI and cTnC was observed, which correlated with the degree of myolysis and calpain activity. Finally, a specific 25kDa degradation product of cTnT observed in PeAF was found to originate from calpain-mediated cleavage. Previously we showed that tachypacing of HL-1 atrial cardiomyocytes induces the prime features of atrial remodeling, including contractile dysfunction, ion-channel and structural remodeling (12,16,17). Our results suggest that calpain activation represents a key factor underlying myofibrillar protein degradation, myolysis and contractile dysfunction. Since myolysis and contractile dysfunction contribute to the AF substrate (1,2), calpain activation may relate to the self-perpetuating nature of AF.

Tachypacing-induced troponin degradation: a central role for calpain

It has been recognized that calcium overload via the L-type calcium channel plays a major role in cardiomyocyte remodeling during AF (19,25). Calcium overload can activate calpain (13) and calpain activation was consistently induced in our cardiomyocyte model for tachypacing-induced remodeling (11,12). In atrial tissue from AF patients, calpain-activity was also significantly elevated (11) and in the present study correlated with the amount of troponin degradation product. Additionally, the resulting calcium overload inactivates L-type calcium channel and affects the calcium-handling apparatus, which lead to secondary abnormalities in calcium-release that result in reduced calcium transient amplitude and consequently decreased CS. Therefore, calcium represents a mediator of tachycardia-induced atrial contractile dysfunction (19). As both tachypacing-induced troponin degradation and contractile dysfunction are attenuated by

the specific calpain inhibitor PD150606, our current data point to a functional relation between the two events, i.e. activation of calpains results in degradation of troponins, which consequently causes contractile dysfunction.

We also investigated two other major protease systems, caspases and the proteasome. While caspases are activated as a result of tachypacing, any role in contractile-protein degradation seems minimal, since effective pan-caspase inhibition did not attenuate cardiac troponin degradation and contractile dysfunction. Although caspases degrade cTnT leading to contractile dysfunction in ventricular cardiomyocytes (22), other studies revealed that calpain, in addition to direct degradation of proteins (13,14), can induce caspase-12 activation, which might result in protein-degradation (26,27). These findings and our observations suggest that upon atrial-tachypacing, calpain specially degrades cardiac troponins, further supporting our hypothesis that calpain and not caspase plays a key role in mediating tachypacing-induced troponin degradation.

The ubiquitin-proteasome system plays an important role in degradation of proteins (24) and promotes cardiac dysfunction in pressure-overloaded hearts (23). In the present study, no role for the proteasome was found in atrial tachypacing-induced degradation of cardiac troponins. This finding agrees with a previous study in which we observed no effect of the proteasome on tachypacing-induced ion-channel remodeling (11).

Collectively, our data indicate that tachypacing of HL-1 atrial cardiomyocytes results in the degradation of troponins, which is mediated by activation of calpain. Although these systems have been implicated previously in AF (8,11), the present study is the first to provide definitive evidence for the role of calpain, and the lack of a role for caspases and proteasome, in troponin degradation and contractile dysfunction in both *in vitro* tachypaced HL-1 atrial cardiomyocytes and human-tissue models for AF. In human AF, we observed degradation of cTnT, cTnI and cTnC only in PeAF patients, compared to PAF and SR. A significant correlation was observed between calpain activity and the amount of degradation product of cTnT and cTnI and a specific cleavage product was

found only in PeAF. A similar cleavage product was observed after tachypacing of human cTnT-transfected HL-1 cardiomyocytes. The appearance of this 25kDa fragment directly results from calpain activation, as its presence was prevented by inhibition of calpains, but not by inhibition of other proteases. Together, the findings suggest that in both human AF and tachypaced HL-1 cardiomyocytes, calpain mediates the degradation of cardiac troponin.

The 25kDa fragment of cTnT was found only in PeAF patients and tachypaced HL-1 cardiomyocytes transfected with human cTnT, but not in untransfected cardiomyocytes. Therefore, the detection of the 25 kDa cleavage product might be interpreted as a possible marker for glyocogen positive and hibernation like cardiomyocytes. The absence of the 25kDa fragment in native HL-1 cells is probably due to differences in N-terminal amino-acid sequence between human and mouse cTnT (28) resulting in the absence of a calpain cleavage site in mouse, since the N-terminal sequence is highly heterogenous.

Implications of calpain activation for AF

It is commonly recognized that AF induces changes at the structural level, predominantly myolysis, which contributes to contractile dysfunction and the progression of AF (4,5,29,30). Our *in vitro* findings combined with human AF data indicate that calpain activation and troponin degradation are closely associated with persistent AF. This observation suggests that Ca^{2+}-overload and subsequent calpain-activation play a role in the self-perpetuation of AF. As cardiac troponin is an important protein-complex of the thin filament to regulate contraction (22,31-33), calpain-mediated troponin degradation will affect contractile function and might therefore be involved in the self-perpetuation of AF. This observation is in line with calpain-induced troponin degradation and the progression of heart failure (34). Furthermore, atrial hypocontractility plays an important role in the thromboembolic complications associated with AF (35). Thus, calpain may be a therapeutic target in cardiac disease.

Since cTnT and cTnI are structurally different from the corresponding forms in skeletal muscle, measurement of serum cTnT and cTnI in patients with heart diseases is recognized to be clinically important in the prediction of severity, prognosis and treatment selection (36). However, so far cardiac troponin is not used as a biomarker in patients with AF. Our study suggests the possibility that the serum concentrations of troponin degradation-products may be applicable as a biomarker in AF patients.

Acknowledgements

We thank Dr. Dirkjan Masman for critical comments on the manuscript and Michel Vos and Jurre Hageman for excellent assistance with plasmid construction. This study was financed by the Dutch Organization for Scientific Research (NWO program grant 916. 46. 043), Ubbo Emmius Bursaal grant from the University of Groningen (Number 800403), Angteq/Kop grant (H2514NL00), the Canadian Institutes of Health Research, the Quebec Heart and Stroke Foundation and the Mathematics of Information Technology and Complex Systems (MITACS) Network.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2008.08.012.

References

1. Nattel S. New ideas about atrial fibrillation 50 years on. *Nature* **2002**;415:219-226.

2. Todd DM, Fynn SP, Walden AP, Hobbs WJ, Arya S, Garratt CJ. Repetitive 4-week periods of atrial electrical remodeling promote stability of atrial fibrillation: time course of a second factor involved in the self-perpetuation of atrial fibrillation. *Circulation* **2004**;109:1434-1439.

3. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation* **1995**;92:1954-1968.

4. Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation* **1997**;96:3157-63.

5. Ausma J, van der Velden HM, Lenders MH, van Ankeren EP, Jongsma HJ, Ramaekers FC *et al.* Reverse structural and gap-junctional remodeling after prolonged atrial fibrillation in the goat. *Circulation* **2003**;107:2051-2058.

6. Bito V, Heinzel FR, Weidemann F, Dommke C, van d, V, Verbeken E *et al.* Cellular mechanisms of contractile dysfunction in hibernating myocardium. *Circ Res* **2004**;94:794-801.

7. Schotten U, Ausma J, Stellbrink C, Sabatschus I, Vogel M, Frechen D *et al.* Cellular mechanisms of depressed atrial contractility in patients with chronic atrial fibrillation. *Circulation* **2001**;103:691-698.

8. Goette A, Arndt M, Rocken C, Staack T, Bechtloff R, Reinhold D *et al.* Calpains and cytokines in fibrillating human atria. *Am J Physiol Heart Circ Physiol* **2002**;283:H264-H272.

9. Thijssen VL, Ausma J, Gorza L, van d, V, Allessie MA, Van G, I et al. Troponin I isoform expression in human and experimental atrial fibrillation. *Circulation* **2004**;110:770-775.

10. Kobayashi T, Solaro RJ. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol* **2005**;67:39-67.

11. Brundel BJ, Ausma J, van Gelder IC, Van der Want JJ, van Gilst WH, Crijns HJ *et al.* Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res* **2002**;54:380-389.

12. Brundel BJ, Kampinga HH, Henning RH. Calpain inhibition prevents pacing-induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. *Cardiovasc Res* **2004**;62:521-528.

13. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* **2003**;83:731-801.

14. Barta J, Toth A, Edes I, Vaszily M, Papp JG, Varro A *et al.* Calpain-1-sensitive myofibrillar proteins of the human myocardium. *Mol Cell Biochem* **2005**;278:1-8.

15. Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A *et al.* HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U A* **1998**;95:2979-2984.

16. Brundel BJ, Shiroshita-Takeshita A, Qi X, Yeh YH, Chartier D, van Gelder IC *et al.* Induction of heat shock response protects the heart against atrial fibrillation. *Circ Res* **2006**;99:1394-1402.

17. Brundel BJ, Henning RH, Ke L, van Gelder IC, Crijns HJ, Kampinga HH. Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation. *J Mol Cell Cardiol* **2006**;41:555-562.

18. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* **2000**;18:538-543.

19. Sun H, Chartier D, Leblanc N, Nattel S. Intracellular calcium changes and tachycardia-induced contractile dysfunction in canine atrial myocytes. *Cardiovasc Res* **2001**;49:751-761.

20. Gallagher MM, Camm AJ. Classification of atrial fibrillation. *Pacing Clin Electrophysiol* **1997**;20:1603-605.

21. Brundel BJ, van Gelder IC, Henning RH, Tuinenburg AE, Wietses M, Grandjean JG *et al.* Alterations in potassium channel gene expression in atria of patients with persistent and paroxysmal atrial fibrillation: differential regulation of protein and mRNA levels for K+ channels. *J Am Coll Cardiol* **2001**;37:926-932.

22. Communal C, Sumandea M, de Tombe P, Narula J, Solaro RJ, Hajjar RJ. Functional consequences of caspase activation in cardiac myocytes. *Proc Natl Acad Sci USA* **2002**;99:6252-6256.

23. Depre C, Wang Q, Yan L, Hedhli N, Peter P, Chen L *et al.* Activation of the cardiac proteasome during pressure overload promotes ventricular hypertrophy. *Circulation* **2006**;114:1821-1828.

24. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **2002**;82:373-428.

25. Ausma J, Dispersyn GD, Duimel H, Thone F, Ver Donck L, Allessie MA *et al.* Changes in ultrastructural calcium distribution in goat atria during atrial fibrillation. *J Mol Cell Cardiol* **2000**;32:355-64.

26. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by

calpain in apoptosis. *J Cell Biol* **2000**;150:887-894.

27. Bajaj G, Sharma RK. TNF-alpha-mediated cardiomyocyte apoptosis involves caspase-12 and calpain. *Biochem Biophys Res Commun* **2006**;345:1558-1564.

28. Zhang Z, Biesiadecki BJ, Jin JP. Selective deletion of the NH2-terminal variable region of cardiac troponin T in ischemia reperfusion by myofibril-associated mu-calpain cleavage. *Biochemistry* **2006**;45:11681-11694.

29. Allessie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. *Cardiovasc Res* **2002**;54:230-246.

30. Thijssen VL, Ausma J, Liu GS, Allessie MA, van Eys GJ, Borgers M. Structural changes of atrial myocardium during chronic atrial fibrillation. *Cardiovasc Pathol* **2000**;9:17-28.

31. Foster DB, Noguchi T, VanBuren P, Murphy AM, Van Eyk JE. C-terminal truncation of cardiac troponin I causes divergent effects on ATPase and force: implications for the pathophysiology of myocardial stunning. *Circ Res* **2003**;93:917-924.

32. Gao WD, Atar D, Liu Y, Perez NG, Murphy AM, Marban E. Role of troponin I proteolysis in the pathogenesis of stunned myocardium. *Circ Res* **1997**;80:393-399.

33. Van Eyk JE, Powers F, Law W, Larue C, Hodges RS, Solaro RJ. Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. *Circ Res* **1998**;82:261-271.

34. Van der Laarse A. Hypothesis: troponin degradation is one of the factors responsible for deterioration of left ventricular function in heart failure. *Cardiovasc Res* **2002**;56:8-14.

35. Khairy P, Nattel S. New insights into the mechanisms and management of atrial fibrillation. *CMAJ* **2002**;167:1012-1020.

36. Wu AH. Cardiac troponin: friend of the cardiac physician, foe to the cardiac patient? *Circulation* **2006**;114:1673-1675.

CHAPTER 3

Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation

Bianca J.J.M. Brundel [a], Robert H. Henning [b], Lei Ke [a], Isabelle C. van Gelder [c], Harry J.G.M. Crijns [d], Harm H. Kampinga [a]

[a] Department of Radiation and Stress Cell Biology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[b] Department of Clinical Pharmacology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, The Netherlands
[c] Department of Cardiology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center of Groningen, The Netherlands
[d] Department of Cardiology, University of Maastricht, The Netherlands

J Mol Cell Cardiol. 2006 Nov;41(3):555-562.

Abstract

Atrial fibrillation (AF) causes myocyte stress by inducing structural changes, predominantly myolysis, which is related to the progression of AF. As heat shock proteins (Hsp) protect against cellular stress, their efficacy in preventing myolysis was investigated in a tachy-paced cell model for AF and in patients with AF. HL-1 atrial myocytes were subjected to tachy-pacing, which induced myolysis. Hsp overexpression was accomplished by a mild heat shock or by the drug geranylgeranylacetone (GGA). Hsp-gene-transfection studies were carried out to investigate roles of individual Hsp. In left and/or right atrial appendages from patients with paroxysmal (n=14), persistent (n=17) AF and controls (n=13) in sinus rhythm (SR), Hsp levels (Westerns) and localization (confocal microscopy) were determined. Heat shock and GGA administered prior to tachy-pacing resulted in almost complete protection against tachy-pacing-induced myolysis. Overexpression of Hsp27, but not of Hsp70, also provided complete protection against pacing-induced myolysis. In patients with paroxysmal AF, Hsp27 expression was significantly increased compared to SR and persistent AF. No changes in Hsp40, Hsc70, Hsp70 and Hsp90 expression levels were observed. Hsp27 levels correlated inversely with the duration of paroxysmal and persistent AF and the extent of myolysis. Furthermore, Hsp27 was localized on myofibrils in tachypaced HL-1 myocytes and in human cardiomyocytes. These data demonstrate that upregulation of Hsp, especially Hsp27, protects tachy-paced atrial myocytes from myolysis. Therefore, the observed elevated Hsp27 expression in patients with paroxysmal AF might serve to protect myocytes from myolysis and limit the progression to persistent AF. Pharmacological induction of Hsp, with drugs such as GGA, may represent a novel therapeutic approach in AF.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia which has the tendency to become more persistent over time (1). Research exploring the underlying mechanisms of the self-perpetuation of AF has demonstrated that AF leads to shortening of atrial effective refractory periods (AERP), heterogeneity of the electrical activation pattern and loss of contractile function (2). When the arrhythmia continues, AF induces changes at the structural level, predominantly myolysis, which are associated with the progression of AF (3-8).

Myolysis is characterized by disruption of the myofibril structure (3,9,10) and observed after various forms of cell stress such as ischemic stress (11) and hypoxia (12). Furthermore, it is well known that some Hsps, especially Hsp70 and Hsp27, protect against the degradation of myofibrils. Hsp70 is able to bind and protect microtubule network and therefore limit myofibril disruption after ischemic stress in myocardium (13). Also, in cells overexpressing Hsp27, F-actin and myofibril structure are protected against heat- or ischemia-induced disruption (14-18) and recovery after disruption is accelerated (19). Because of these protective roles of Hsp, we studied whether upregulation of Hsps prior to and during pacing can protect against tachypacing-induced myolysis in an established cell model for AF (20,21). In this model, induction of Hsp synthesis was performed by a mild heat shock or by the drug geranylgeranylacetone (GGA). GGA, clinically employed as an antiulcer agent, is a non-toxic acyclic isoprenoid compound with a retinoid skeleton that induces Hsp synthesis in various tissues including gastric mucosa, intestine, liver, myocardium, retina and central nervous system under stress (22-24). In the present study, we found that Hsp upregulation protects against pacing-induced myolysis. Notably, the sole overexpression of one member of Hsp, Hsp27, was sufficient for this protection.

Materials and Methods

HL-1 cell culture conditions, transfections and constructs

The HL-1 atrial myocytes, developed from adult mouse atria (25), were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as described before (20).

Lipofectamine (Life technologies, The Netherlands) was used for transient transfections according to instructions of the manufacturer. pHsp70-YFP encodes a functional human Hsp70 fused to YFP under control of a CMV promoter. pHsp27 encodes human Hsp27 under control of CMV promoter (Clontech, The Netherlands). Hsp27 transfected myocytes were detected by immunofluorescent staining with the human specific Hsp27 antibody (SPA800, StressGen Biotechnologies, Victoria, Canada) and myocytes overexpressing human Hsp70-YFP were distinguished from non-transfected myocytes on the basis of the YFP signal.

Pacing and induction of Hsp expression

HL-1 myocytes ($\geqslant 1\times10^6$ myocytes) were cultured on coverslips and subjected to a 10-fold rate increase (tachypacing) by the use of a C-Pace100™-culture pacer and CDish100™-culture dishes (IonOptix Corporation, The Netherlands). Myocytes were stimulated at 5 Hz with square pulses of 5 ms duration and a pulse voltage set to 45 V. Evidence of capture was examined by microscopic examination of cell shortening at the beginning and before termination (after 24 h pacing) of stimulation. Capture efficiency at these time points was >90% for myocytes attached on gelatin/fibronectin-coated coverslips. Furthermore, the role of non-specific factors, such as electrolysis at the electrodes, was excluded by placing control myocytes outside the electrical field by the use of a specially developed pacing system. In this system, coverslip-cultured myocytes can be placed either within the electric field or outside the electric field. Control

myocytes placed outside the electric field showed no signs of myocyte remodeling, whereas myocytes placed in the electric field revealed myocyte remodeling (20).

Elevation of Hsp expression in cultured myocytes was accomplished in 3 ways: (I) by subjection to a modest heat shock at 43 °C for 30 min followed by overnight incubation at 37 °C, (II) by incubation with 0.1 μ M geranylgeranylacetone (GGA, gift from Eisai Co. Japan) 2 h prior to and during pacing and (III) by transfection of pHsp70-YFP or pHsp27 24 h prior to pacing. The concentration-dependent increase in Hsp expression by GGA was tested at 0.1, 1 and 10 μ M. Since 90% of the maximum effect on induction of Hsp expression was already reached at 0.1 μ M GGA, all experiments were performed at this concentration.

Patients

Prior to surgery, one investigator assessed the clinical characteristics of the patients (Table 1). The patient's arrhythmia history was classified according to Gallagher (26). The persistent (n=17) and paroxysmal (n=14) group contained patients with lone AF or AF with underlying mitral valve disease (MVD). All patients were euthyroid and had normal left ventricular function. Coumarin therapy was interrupted 3 days before surgery and class I and III antiarrhythmic drugs were discontinued for at least 5 half times. Right and/or left atrial appendages (RAAs and LAAs, respectively) were obtained from all patients, except for the control patients undergoing CABG from whom only the RAA was gathered prior to cardiopulmonary bypass (Table 1). After excision, the atrial appendages were immediately snap-frozen in liquid nitrogen and stored at -85 °C. The Institutional Review Board approved the study and patients gave written informed consent.

Table 1 Baseline characteristics of patients with paroxysmal AF (PAF), persistent AF (PeAF) and control patients in sinus rhythm

	SR	PAF	PeAF
n	13	14	17
RAA (*n*)	11	12	16
LAA (*n*)	5	12	16
Age	61±4	50±3	53±3
Duration of AF (median, range (months))	–	–	11.6 (0.1–56)
Duration SR before surgery (median, range (days))	–	10 (0.5–210)	–
Duration of last episode AF (median, range (h))	–	12 (0.2–24)	–
AF/day (median, range (%))	–	2 (0.2–70)	–
Underlying heart disease (*n*) and/surgical procedure			
Coronary artery disease/CABG	8*	0	0
Lone AF/Maze	0	8	9
MVD/MV replacement/repair	5	6	8
New York Heart Association for exercise tolerance			
Class I	10*	6	5
Class II	3	5	8
Class III	0	3	4
Systolic blood pressure	147±4	141±9	135±7
Diastolic blood pressure	84±2	81±3	86±2
Echocardiography			
Left atrial diameter (parasternal)	42±3	42±4	48±4
Left ventricular end-diastolic diameter (mm)	50±4	52±3	52±3
Left ventricular end-systolic diameter (mm)	34±4	38±3	34±5
Medication (*n*)			
Ace-inhibitors	4	5	7
Digitalis	0	1	7*
Verapamil	6	3	4
Beta-blocker	4	3	4
Hsp/GAPDH protein ratio			
Hsp27	0.7±0.05	1.2±0.07*	0.8±0.08
Hsp40	1.2±0.2	1.4±0.3	1.4±0.2
Hsc70	0.7±0.2	0.8±0.2	0.7±0.1
Hsp70	0.6±0.2	1.0±0.3	0.7±0.3
Hsp90	1.2±0.3	1.1±0.6	1.3±0.4

Values are presented as mean value±SEM or number of patients. CABG: Coronary Artery Bypass Grafting; Maze: atrial arrhythmia surgery; MVD: mitral valve disease. *$p<0.05$.

Protein extraction and Western blot analysis

For Western blot analysis, frozen RAAs and LAAs were used for protein isolation as described previously (27). For the isolation of proteins from HL-1 myocytes, the cells were lysed by the addition of SDS-PAGE sample buffer followed by sonication before separation on 10% PAA-SDS gels (1.10^5 cells/slot). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against GAPDH (Affinity Reagents, USA), rodent Hsp25 (SPA801), human Hsp27 (SPA800), Hsp40 (SPA400), Hsc70 (SPA815), Hsp70 (SPA810) or Hsp90 (SPA835) (all StressGen Biotechnologies, Victoria, Canada). Horseradish peroxidase-conjugated anti-mouse, anti-rat or anti-rabbit IgG (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry. The amount of protein chosen was in the linear immunoreactive signal range and expressed relative to GAPDH.

Immunofluorescent staining, quantification and confocal analysis

After subjecting HL-1 myocytes to tachy-pacing, the cells were fixed for 10 min in 100% methanol (-20 °C), dried and blocked in 5% BSA (20 min room temperature). Antibodies against myosin heavy chain (MF-20, Developmental Studies Hybridoma Bank, Baltimore, MD, USA) or Hsp27 (StressGen Biotechnologies, Vicotria, Canada) were used as primary antibody. Fluorescein labeled isothiocyanate (FITC) anti-mouse and anti-rabbit (Jackson ImmunoResearch, The Netherlands) or N, N' (dipropyl) –tetramethyl -indocarbocyanine Cy3 anti-mouse (Amersham, The Netherlands) was used as secondary antibody. Nuclei were visualized by 4', 6-diamidino-2-phenylindole (DAPI) staining. Images of FITC, YFP or CY3 and DAPI fluorescence were obtained by using a Leica confocal laser-scanning microscope (Leica TCS SP2).

For the quantification of the amount of myolysis, 5 fields or more were examined with a

total amount of 250 - 500 myocytes, and myosin disruption (characteristic for myolysis (3)) was scored by three independent observers blinded for the experimental groups. An atrial myocyte was defined as myolytic when >10% of the myocyte surface was free from myosin staining (3,28). Mean scores of the observers were used.

Definitions

Persistent AF

Continuous presence of AF until the moment of cardiac surgery, i.e. at least two consecutive electrocardiograms of AF more than 1 week apart, without intercurrent sinus rhythm. Persistent AF has a non-spontaneously converting character. Previously, this type of AF was classified as chronic AF (29).

Paroxysmal AF

AF typically occurring in episodes with a duration shorter than 24 h (but longer lasting paroxysms are not unusual) with intermittent sinus rhythm. Paroxysmal AF either converts spontaneously or is terminated by intravenously administered antiarrhythmic drug. It is non-controlled whether paroxysmal AF is present at the moment of cardiac surgery (29).

Statistical analysis

Results are expressed as mean±SEM. All Western blot procedures were performed in duplicate series and morphological quantifications were performed in duplo series of at least n=6 wells per series. Mean values were used for statistical analysis. ANOVA was used for multiple group comparisons. Correlation was determined using the Spearman correlation test. All p values were two-sided, a p value of <0.05 was considered statistically significant. SPSS version 8.0 was used for all statistical evaluations.

Results

Hsp protect HL-1 myocytes from myolysis

To address whether Hsp can protect from myolysis induced by AF, we applied a tachy-paced cell model for AF which reveals characteristic features of AF (20), including the induction of myolysis (Fig. 1B). To test whether increased Hsp expression is capable of protecting against tachy-pacinginduced myolysis, myocytes were pretreated with a mild non lethal heat shock (30 min at 43 °C) and tachy-paced from 16 h afterwards. This mild heat shock induced all major heat shock proteins, including Hsp27 (in rodents often referred to as Hsp25) and Hsp70 prior to and during pacing (Fig. 1A). Heat-treated myocytes showed less pacing-induced myolysis than non-pretreated paced myocytes (Figs. 1B, C).

To test whether pharmacological induction of Hsp with the non-toxic drug GGA was able to protect from myolysis, GGA was applied 2 h prior to and during pacing. Pacing in combination with GGA treatment led to significant elevations in Hsp27 and Hsp70 expression (Fig. 1A), which coincided with a significant reduction in pacing-induced myolysis compared to normal paced myocytes (Figs. 1B, D).

Hsp27 overexpression is sufficient for protection from tachypacing-induced myolysis

To conclusively establish whether or not Hsp upregulation directly protects from pacing-induced myolysis and to study which Hsp conveys this protection, HL-1 atrial myocytes were transiently transfected with plasmids either encoding human Hsp27 or human Hsp70. In myocytes transiently overexpressing human Hsp27, as detected by immunofluorescent staining with a human specific Hsp27 antibody (Fig. 2A, left panel), a significant reduction of pacing-induced myolysis compared to normal paced myocytes was found (Fig. 2B). The protection mediated by overexpression of Hsp27 alone was as efficient as seen after heat-shock or GGA treatment. Furthermore, partial colocalization of Hsp27 with myosin was observed (Fig. 2A), suggesting an interaction between Hsp27

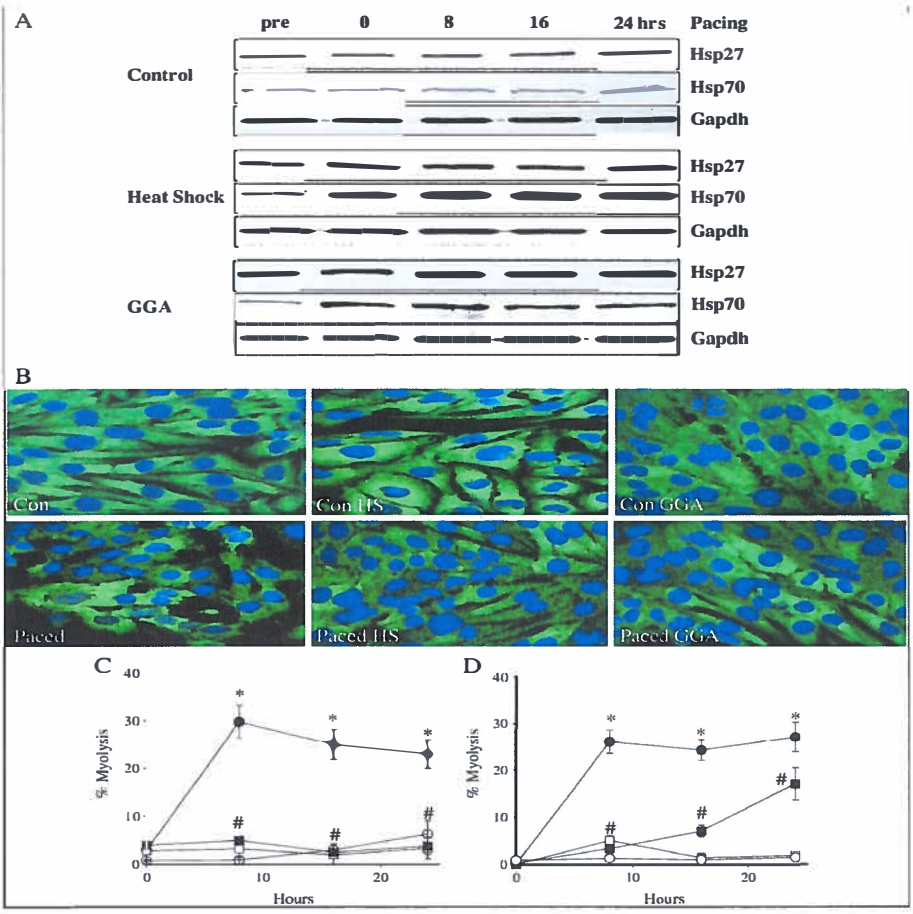


Figure 1 The effect of induction of Hsp levels on pacing-induced myolysis. **(A)** Western blots show that a heat shock (HS) or GGA treatment (GGA) induces the expression of endogenous Hsp27 and Hsp70 in time, but do not change GAPDH levels, compared to non-treated myocytes (control). Increased levels are maintained during pacing. **(B)** Immunofluorescent staining of myosin (green) in non-paced myocytes (Con), heat-shocked control myocytes (Con HS) and GGA-treated control myocytes (Con GGA) compared to 16 h paced myocytes (Paced), paced HS myocytes and paced GGA-treated myocytes. Paced myocytes reveal disruption of myosin (myolysis), whereas myosin staining remains diffusely distributed in the cytoplasm of paced myocytes treated with either HS or GGA. **(C)** Quantification of percentage myocytes showing myolysis as a function of time of pacing in control and heat-shocked myocytes (non-paced myocytes ○, non-paced HS myocytes □, paced myocytes●, paced HS myocytes). **(D)** Quantification of percentage myolysis in GGA-treated myocytes (non-paced control myocytes○, non-paced GGA myocytes □, paced myocytes ● and paced GGA myocytes). *=significant increase compared to non-paced myocytes ($p<0.01$); #=significant reduction compared to paced myocytes ($p\leqslant 0.05$).

and relevant target proteins. In contrast, myocytes transiently overexpressing human Hsp70-YFP, distinguished from non-transfected myocytes on the basis of the YFP signal (Fig. 2A, right panel), were not protected against pacing-induced myolysis (Fig. 2B). Together, the results demonstrate that a general induction of Hsp expression protects against pacing-induced myolysis and that the sole overexpression of Hsp27, but not Hsp70, is sufficient for this protection.

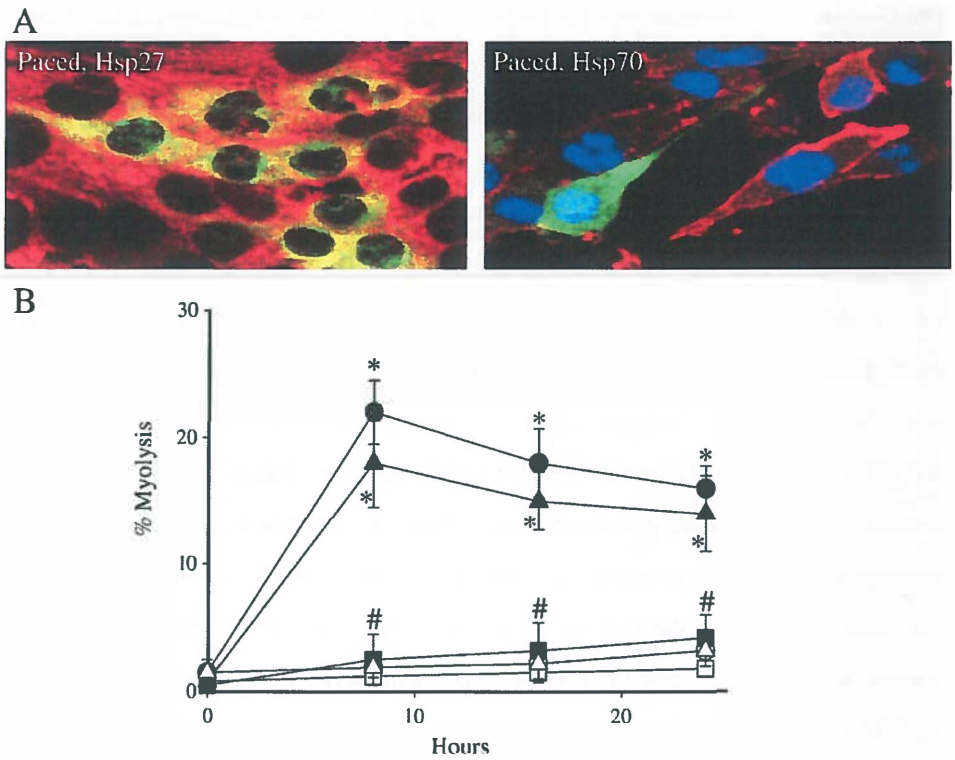


Figure 2 The effect of human Hsp27 or Hsp70 expression on pacing-induced myolysis. **(A)** Immunofluorescent staining of human Hsp27 (green), Hsp70-YFP (green) positive myocytes, myosin (red) and nuclei (DAPI, blue) in 16 h paced myocytes. Co-localization of Hsp27 with myosin was observed (yellow). (B) Quantification of the percentage myolysis in Hsp27 expressing myocytes (paced Hsp27 , non-paced Hsp27 □), Hsp70 expressing myocytes (paced Hsp70 , non-paced Hsp70) and in untransfected control myocytes (paced myocytes ●, non-paced control myocytes ○). *=significant increase compared to non-paced control myocytes ($p<0.01$); #=significant reduction compared to paced control myocytes ($p \leqslant 0.05$).

Hsp expression correlates with duration of persistent AF and structural changes

In extension to the findings in the tachy-paced cell model for AF, Hsp levels in atrial tissue from patients with AF were determined. Protein isolated from atrial appendages was used for immunological detection of Hsp27, Hsp40, Hsc70, Hsp70 and Hsp90. Changes in protein expression were studied in relation to protein levels of GAPDH, which did not differ between the groups (data not shown). The protein expression of Hsp27 (Fig. 3A) was significantly increased in RAAs and LAAs from patients with paroxysmal AF compared to samples from control patients ($p<0.0001$) and patients with persistent AF ($p<0.0001$). A trend for increased Hsp70 expression ($p=0.05$) was observed in patients with paroxysmal AF compared to control patients (Fig. 3B, Table 1). No significant differences in the expression levels of Hsp40, Hsc70 and Hsp90 (Fig. 3B, Table 1) were found between the groups and no significant differences between RAA and LAA within the groups were observed. Also, no significant differences in Hsp27 levels were observed between tissue from lone AF patients and AF patients with underlying valve disease. Since increased Hsp27 levels were only found in tissue from paroxysmal AF patients, this may reflect that Hsps are merely activated in acute stress conditions and become exhausted during prolonged stress (30,31). To test this, a correlation was made between the duration of persistent AF and Hsp27 levels. A significant inverse correlation was observed between the duration of persistent AF and Hsp27 expression (Fig. 3C). Patients with a shorter duration of AF revealed higher levels of Hsp27 expression. Also a significant inverse correlation was observed between the duration of the last episode of AF and the Hsp27 levels in patients with paroxysmal AF (Fig. 3D). Paroxysmal AF patients with short period of AF display higher levels of Hsp27 expression. No significant correlation between Hsp27 expression and age, or medication was observed (data not shown).

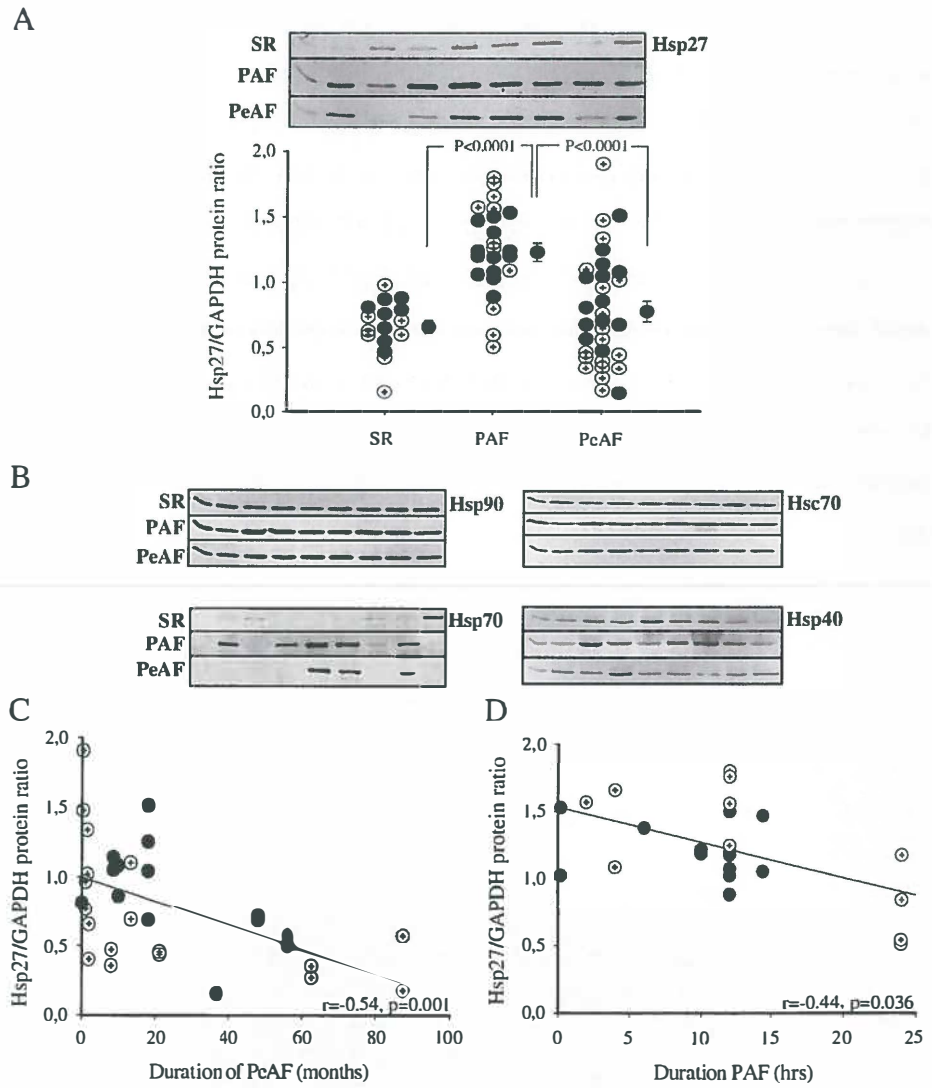


Figure 3 Protein expression levels of Hsp27 (**A**) in atrial tissue of patients with paroxysmal AF (PAF), persistent AF (PeAF) and controls in sinus rhythm (SR). Protein expression levels were determined by Western blotting and expressed as ratios over GAPDH. Inserts show part of typical Western blots. Patients with PAF reveal significant increase in Hsp27 protein ratios compared to controls in sinus rhythm (SR). **(B)** Part of typical Western blots showing Hsp90, Hsp70, Hsc70 and Hsp40 expression levels in SR, paroxysmal AF or persistent AF patients. (**C**) Correlation between Hsp27/GAPDH protein ratio and duration of persistent AF (PeAF). (**D**) Correlation between Hsp27/GAPDH protein ratio and duration of last episode of paroxysmal AF (PAF). (•) Represents lone AF patients or SR patients undergoing CABG, (⊕) patients with AF or SR and mitral valve disease.

Previously, we reported on (ultra)structural changes in atrial tissue of a part of this patient population (28). In brief, in 7 patients with lone persistent AF, a substantial fraction of cells was myolytic (30.0±14.5%), whereas the percentage of myocytes with myolysis in tissue of 6 patients with lone paroxysmal AF was low (6.9±6.1%) and similar to that in control patients (5.5±3.6%). In this sub-group of AF patients, we found an inverse correlation between the amount of myolysis and Hsp27 expression (Fig. 4A), i.e. high Hsp27 levels in tissue of patients were associated with low amounts of myolysis. Furthermore, confocal microscopy revealed that, like in the tachy-paced cell model for AF, Hsp27 was preferably localized on myofibrils in cardiomyocytes (both of SR and AF patients), whereas Hsp70 showed diffuse cytosolic and extracellular matrix staining (Fig. 4B).

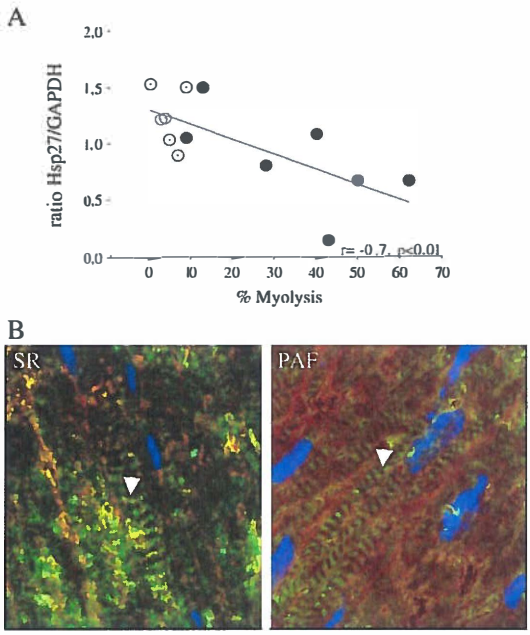


Figure 4 An inverse correlation was found between the extent of myolysis and protein expression levels of Hsp27 **(A)** in RAAs of patients with paroxysmal AF (⊙) and persistent AF (•). **(B)** Typical example of an immunofluorescent staining of Hsp27 (green), Hsp70 (red) and nuclei (DAPI, blue) in atrial tissue of a patient in sinus rhythm (SR) and with paroxysmal AF (PAF). Hsp27 is localized on the myofibrils in cardiomyocytes (arrowhead), whereas Hsp70 is located in the cytosol and extracellular matrix.

Discussion

The present study identifies a protective role for Hsp in AF. Using the HL-1 cell model for AF (20), we provided evidence that upregulation of Hsp by a mild heat shock and pharmacologically by the drug GGA, attenuates myolysis. Furthermore, transfection experiments directly demonstrated that elevated expression of Hsp27 alone was sufficient for this protection. The results were extended to human AF, where a highly significant increase of Hsp27 expression was observed only in atrial appendages of patients with paroxysmal AF. In addition, the expression levels of Hsp27 correlated inversely with the duration of the arrhythmia and with the amount of myolysis in paroxysmal and persistent AF. Finally, like in the tachypaced cell model for AF, Hsp27 was found to be localized at the myofilaments in human atrial myocytes. Our data imply that Hsp upregulation protects against tachypacing-induced myolysis.

Mechanism of Hsp protection

Several mechanisms may explain how Hsp27 protects myocytes from tachypacing -induced myolysis. Pacing directly, or via increases of intracellular free calcium and calpain activation (20,28,32), results in disruption of myofibril structures (3). A first possibility is that Hsp27 binds to the myofibril structure, as suggestive from the (co)localization of Hsp27 with myosin in HL-1 myocytes and at myofilaments in human atrial myocytes. Such binding would be in line with previous studies in human and rat heart (14,17) and suggest that by binding to contractile proteins, Hsp27 stabilizes myofibrils during AF similar to the observed Hsp27-mediated protection of cytoskeletal and contractile elements after heat or ischemic stress in myocardium and smooth muscle cells (15,19,33). Alternatively, binding of Hsp27 to contractile proteins may shield them from cleavage by cystein proteases. Cystein proteases are known to become activated during AF and result in cleavage of myofilamental proteins (20,28,34,35). Finally, Hsp27 may accelerate the recovery of myofilamental proteins after disruption, similar to its

action on accelerating actinrepolymerization (19).

Hsp27 expression in paroxysmal and persistent AF

In atrial tissue of AF patients, Hsp27 expression was only significantly elevated in atrial tissue from paroxysmal AF patients and not in persistent AF patients. Moreover, in paroxysmal and persistent AF, the variation in Hsp27 levels was large and found to be inversely correlated with duration of AF and with the amount of myolysis. Generally speaking, upregulation of heat shock proteins in response to a stressful event depends both on the intensity and duration of the stress. While it is known that the magnitude of the response is dependent on the degree of stress (36), inevitably, the response gets exhausted in time if the stress continues (37). Given these features of the heat shock response and the antimyolytic effect of Hsp27 in tachy-paced myocytes, the patient data may be interpreted as follows. For intermittent periods of stress, such as paroxysmal AF in which arrhythmias are separated by non-stressful intervals of normal sinus rhythms, myocytes are capable of increasing Hsp levels, which might prevent myofibril disruption. As a result, patients with paroxysmal AF may be able to overcome AF paroxysms without the induction of structural changes such as myolysis. The observed inverse correlation between Hsp27 levels and duration of persistent AF and paroxysmal AF suggests that the heat shock response gets exhausted in time, leading to a loss of its protective effects, thereby promoting the progression to persistent AF. Indeed, it is shown that the heat shock response gets temporarily activated in acute but not in chronic diseases (30), during cardiac differentiation (38), and it attenuates with age (37).

In our study, patients in sinus rhythm with underlying coronary artery disease or mitral valve disease were used. Previously, it was described that Hsp27 levels in failing hearts were significantly increased compared to normal hearts (39). This would suggest that the, in the present study, observed increase in Hsp27 levels in patients with paroxysmal AF compared to control patients in sinus rhythm but without underlying heart diseases would

be even more significant. Furthermore, the NYHA class I was significantly different in both patients with paroxysmal and persistent AF compared to SR patients, and also more patients with persistent AF used digitalis compared to paroxysmal AF and SR patients. These differences could potentially have influenced the results on myolysis and Hsp levels.

In summary, the results from both the tachypaced cell model for AF and human AF provide evidence that elevated expression of Hsp27 protects myocytes from tachypacing induced myolysis. The Hsp response that gets temporarily activated in patients with AF, seems to exhaust in time, thereby losing the ability to prevent structural changes like myolysis, thus leading to the progression of AF. Future experiments are therefore warranted to identify the therapeutic usefulness of drugs that boost the Hsp response, such as GGA, in AF.

Acknowledgments

This study was financed by the European Union (QLK3-CT-2000-00720) and the Dutch Organization for Scientific Research (NWO program grant 916.46.043). We want to thank Maria de Boer, Lybrich Schakel and Willy Lemstra-Wierenga for excellent technical assistance and Eisai Co. Japan for providing GGA.

References

1. Godtfredsen J. Etiology, course and prognosis. A follow-up study of 1212 cases. Copenhagen: University of Copenhagen; **1975**.

2. Nattel S. New ideas about atrial fibrillation 50 years on. *Nature* **2002**; 415:219-226.

3. Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation* **1997**;96:3157-3163.

4. Allessie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. *Cardiovasc Res* **2002**;54:230-246.

5. Thijssen VLJL, Ausma J, Liu GS, Allessie M, Eys GJJM, Borgers M. Structural changes of atrial myocardium during chronic atrial fibrillation. *Cardiovasc Pathol* **2000**;9:17-28.

6. Ausma J, Van der Velden HMW, Lenders MH, Van Ankeren EP, Jongsma HJ, Ramaekers F, *et al.* Reverse structural and gap-junctional remodeling after prolonged atrial fibrillation in the goat. *Circulation* **2003**;107:2051-2058.

7. Verheule S, Sato T, Everett T, Engle SK, Otten D, Rubert-von der Lohe M, *et al.* Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-b1. *Circ Res* **2004**;94:1458-1465.

8. Todd DM, Fynn SP, Walden AP, Hobbs WJ, Arya S, Garratt CJ. Repetitive 4-week periods of atrial electrical remodeling promote stability of atrial fibrillation: time course of a second factor involved in the self-perpetuation of atrial fibrillation. *Circulation* **2004**;109:1434-1439.

9. Vanoverschelde JLJ,WijnsW, Depré C, Essamri B, Heyndricks S, Borgers M, *et al.* Mechanisms of chronic regional postischemic dysfunction in humans: new insights from the study on non-infarcted collateral dependent myocardium. *Circulation* **1993**;87:1513-1523.

10. Allessie MA, Boyden PA, Camm AJ, Kléber AG, Lab MJ, Legato MJ, *et al.* Pathophysiology and prevention of atrial fibrillation. *Circulation* **2001**;103:769-777.

11. Thomas SA, Fallavollita JA, Suzuki G, Borgers M, Canty JM. Dissociation of regional adaptations to ischemia and global myolysis in an accelerated swine model of chronic hibernating myocardium. *Circ Res* **2004**;91:970-977.

12. Maes A, Flameng W, Nuyts J, Borgers M, Shivalkar B, Ausma J, *et al.* Histological alterations in chronically hypoperfused myocardium. Correlation with PET findings. *Circulation* **1994**;90:735-745.

13. Decker RS, Decker ML, Nakamura S, Zhao YS, Hedjbeli S, Harris KR, Klocke FJ. Hsc73 - tubulin complex formation during low-flow ischemia in canine myocardium. *Am J Physiol Heart Circ Physiol* **2002**;283:H1322-333.

14. Lutsch G, Vetter R, Offhauss U, Wieske M, Gröne HJ, Klemenz R, *et al.* Abundance and location of the small heat shock proteins Hsp25 and aBcrystallin in rat and human heart. *Circulation* **1997**;96:3466-3476.

15. Bryantsev AL, Loktionova SA, Ilyinskaya OP, Tararak EM, Kampinga HH, Kabakow AE. Distribution, phosphorylation, and activities of Hsp25 in heat-stressed H9c2 myoblasts: a functional link to cytoprotection. *Cell Stress Chaperones* **2002**;7:146-155.

16. Van den Klundert M, Gijsen ML, van den IJssel PR, Snoeckx LH, de Jong WW. Alpha B-crystallin and hsp25 in neonatal cardiac cells—Differences in cellular localization under stress conditions. *Eur J Cell Biol* **1998**;75:38-45.

17. Hoch B, Lutsch G, Schlegel WP, Stahl J, Wallukat G, Bartel S, *et al.* HSP25 in isolated perfused rat hearts: localization and response to hyperthermia. *Mol Cell Biochem* **1996**;160-161:231-239.

18. Sanada S, Kitakaze M, Papst PJ, Hatanaka K, Asanuma H, Aki T, *et al.* Role of phasic dynamism of p38 mitogen-activated protein kinase activation in ischemic preconditioning of the canine heart. *Circ Res* **2001**;88:175-180.

19. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol* **1995**;15:505-516.

20. Brundel BJJM, Kampinga HH, Henning RH. Calpain inhibition prevents pacing induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. *Cardiovasc Res* **2004**;62:521-528.

21. Yang Z, Shen W, Rottman JN, Wikswo JP, Murray KT. Rapid stimulation causes electrical remodeling in cultured atrial myocytes. *J Mol Cell Cardiol* **2005**;38:299-308.

22. Hirakawa T, Rokutan K, Nikawa T, Kishi K. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* **1996**;111:345-357.

23. Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, et al. Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. *Circulation* **2001**;104:1837-1843.

24. Katsuno M, Sang C, Adachi H, Minamiyama M, Waza M, Tanaka F, *et al.* Pharmacological induction

of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. *Proc Natl Acad Sci USA* **2005**;102:16801-16806.

25. Claycomb WC, Lanson NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, *et al.* HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA* **1998**;95:2979-2984.

26. Gallagher MM, Camm AJ. Classification of atrial fibrillation. *Pacing Clin Electrophysiol* **1997**;20:1603-1605.

27. Brundel BJJM, Van Gelder IC, Henning RH, Tuinenburg AE, Tieleman RG, Wietses M, *et al.* Ion channel remodeling is related to intra-operative atrial refractory periods in patients with paroxysmal and persistent atrial fibrillation. *Circulation* **2001**;103:684-690.

28. Brundel BJJM, Ausma J, Van Gelder IC, Van DerWant JJL, Van Gilst WH, Crijns HJGM, *et al.* Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res* **2002**;54:380-389.

29. Brundel BJJM, Van Gelder IC, Henning RH, Tuinenburg AE, Deelman LE, Tieleman RG, *et al.* Gene expression of proteins influencing the calcium homeostasis in patients with persistent and paroxysmal atrial fibrillation. *Cardiovasc Res* **1999**;42:443-454.

30. Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L. Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. *Nat Med* **2004**;10:402-405.

31. Soti C, Nagy E, Giricz Z, Vigh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol* **2005**;146:769-780.

32. Sun H, Chartier D, Leblanc N, Nattel S. Intracellular calcium changes and tachycardia-induced contractile dysfunction in canine atrial myocytes. *Cardiovasc Res* **2001**;49:751-761.

33. Somara S, Bitar KN. Tropomyosin interacts with phosphorylated Hsp27 in agonist-induced contraction of smooth muscle. *Am J Physiol Cell Physiol* **2004**;286:C1290-1301.

34. Moretti A, Weig HJ, Ott T, Seyfarth M, Holthoff HP, Grewe D, *et al.* Essential myosin light chain as a target for caspase-3 in failing myocardium. *Proc Natl Acad Sci USA* **2002**;99:11860-11865.

35. Communal C, Sumandea M, De Tombe P, Narula J, Solaro RJ, Hajjar RJ. Functional consequences of caspase activation in cardiac myocytes. *Proc Natl Acad Sci USA* **2002**;99:6252-6256.

36. Kampinga HH. Thermotolerance in mammalian cells. Protein denaturation and aggregation, and stress proteins. *J Cell Sci* **1993**;104:11-17.

37. Demirel HA, Hamilton KL, Shanely RA, Tumer N, Koroly MJ, Powers SK. Age and attenuation of exercise-induced myocardial HSP72 accumulation. *Am J Physiol Heart Circ Physiol* **2003**; 285: H1609-1615.

38. Benjamin IJ, Shelton J, Garry DJ, Richardson JA. Temporospatial expression of the small HSP/alpha B-crystallin in cardiac and skeletal muscle during mouse development. *Dev Dyn* **1997**;208:75-84.

39. Knowlton AA, Kapadia S, Torre-Amione G, Durand JB, Bies R, Young J,*et al*. Differential expression of heat shock proteins in normal and failing human hearts. *J Mol Cell Cardiol* **1998**;30:811-818.

CHAPTER 4

HSPB1, HSPB6, HSPB7 and HSPB8 protect against RhoA GTPase-induced remodeling in tachypaced atrial myocytes

Lei Ke MD[a*], Roelien A.M. Meijering MSc[b*], Femke Hoogstra-Berends BSc[b,c], Katarina Mackovicova MSc[b], Michel J. Vos PhD[a], Isabelle C. Van Gelder MD PhD[d], Robert H. Henning MD PhD[b], Harm H. Kampinga PhD[a], Bianca J J.M. Brundel PhD[b]

[a] Department of Radiation and Stress Cell Biology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands

[b] Department of Clinical Pharmacology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands

[c] Nyken BV, Groningen, The Netherlands

[d] Department of Cardiology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, and the Interuniversity Cardiology Institute Netherlands, Utrecht, The Netherlands

* Both authors contributed equally to this work.

PLoS One. 2011;6(6):e20395

Abstract

Background: We previously demonstrated the small heat shock protein, HSPB1, to prevent tachycardia remodeling in *in vitro* and *in vivo* models for Atrial Fibrillation (AF). To gain insight into its mechanism of action, we examined the protective effect of all 10 members of the HSPB family on tachycardia remodeling. Furthermore, modulating effects of HSPB on RhoA GTPase activity and F-actin stress fiber formation were examined, as this pathway was found of prime importance in tachycardia remodeling events and the initiation of AF.

Methods and Results: Tachypacing (4Hz) of HL-1 atrial myocytes significantly and progressively reduced the amplitude of $Ca2^{+}$ transients (CaT). In addition to HSPB1, also overexpression of HSPB6, HSPB7 and HSPB8 protected against tachypacing-induced CaT reduction. The protective effect was independent of HSPB1. Moreover, tachypacing induced RhoA GTPase activity and caused F-actin stress fiber formation. The ROCK inhibitor Y27632 significantly prevented tachypacing-induced F-actin formation and CaT reductions, showing that RhoA activation is required for remodeling. Although all protective HSPB members prevented the formation of F-actin stress fibers, their mode of action differs. Whilst HSPB1, HSPB6 and HSPB7 acted via direct prevention of F-actin formation, HSPB8-protection was mediated via inhibition of RhoA GTPase activity.

Conclusion: Overexpression of HSPB1, as well as HSPB6, HSPB7 and HSPB8 independently protect against tachycardia remodeling by attenuation of the RhoA GTPase pathway at different levels. The cardioprotective role for multiple HSPB members indicates a possible therapeutic benefit of compounds able to boost the expression of single or multiple members of the HSPB family.

Introduction

Atrial Fibrillation (AF) is the most common sustained and progressive clinical tachycardia in the population and it significantly contributes to cardiovascular morbidity and mortality (1). AF is characterized by specific changes in electrical, structural and conctractile function of the atrial myocytes, commonly denoted as 'remodeling'. Tachycardia remodeling underlies contractile dysfunction and the progressive and intractable nature of AF. Therefore, remodeling is believed to have important therapeutic implications, and there is great interest in developing anti-remodeling therapies directed at the targets underlying remodeling (1).

We recently identified one specific member of the heat shock protein (HSP) family, HSPB1, to protect against AF-induced remodeling (2,3). HSPs are molecular chaperones and prevent the accumulation of the misfolded or unfolded proteins in the cells (4). HSPB1 is one member of the small heat shock protein (sHSP or HSPB in mammals) family, which comprises a total of at least ten members (5,6). A characteristic of most HSPBs is their ability to interact with components of the actin cytoskeleton, and this binding protects against cytoskeletal injury during stress, resulting in conservation of the cell function (7). In addition, HSPBs collectively share important features, including <1> a conserved α-crystallin domain, <2> ability to form large oligomers *in vitro* and <3> increased expression upon exposure to various stresses including heat stress (8). Nevertheless, the precise mode of action of HSPB1 to protect from tachycardia remodeling remains elusive and it is unknown whether this is shared between other members of the HSPB family. Therefore, we examined if, in addition to HSPB1, also other HSPB members protect against atrial tachycardia remodeling. Hereto, we utilized tachypaced HL-1 myocytes, an *in vitro* atrial cell line model for tachycardia remodeling (3,9). In addition to HSPB1, we identified HSPB6, HSPB7 and HSPB8 to protect against tachypacing-induced calcium transient reduction. Because of the known protective actions of HSPBs on actin cytoskeleton, we next examined their effect on

tachypacing-induced RhoA GTPase pathway, including RhoA GTPase activity and related F-actin stress fiber formation. Although all protective HSPB members reduced the formation of F-actin stress fibers, their mode of action differs. HSPB1, HSPB6 and HSPB7 were found to directly prevent F-actin stress fiber formation, whereas HSPB8-protection was mediated via inhibition of upstream RhoA GTPase activity.

Materials and Methods

HL-1 atrial myocyte culture, transfections and constructs

HL-1 atrial myocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb as described before (3). The myocytes were maintained in Complete Claycomb Medium (JRH, UK) supplemented with 100 μM norepinephrine (Sigma, The Netherlands), 0.3 mM L-ascorbic acid (Sigma), 4 mM L-glutamine (Gibco, The Netherlands) and 10% FBS (Life Technologies, Gaithersburg, MD). They were cultured on coverslips coated with 12.5 μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma), in a 5% CO_2 atmosphere at 37° C.

To study the influence of HSPBs on Ca^{2+} transient changes, HL-1 myocytes were transiently (co-)transfected, by the use of Lipofectamin (Life Technologies, The Netherlands), with the plasmid CD8 cDNA encoding CD8 antigen and/or pCDNA5/FRT/TO-HSPBX (X indicating 1-10) encoding human HSPB members. Positive myocytes were selected by anti-CD8 Dynabeads (Dynal). To check overexpression of HSPBX proteins in HL-1 myocytes, myocytes were transiently transfected with the fusion proteins V5-HSPBX. For all other experiments HSPBX wildtype constructs were used.

Tachypacing of HL-1 myocytes

HL-1 myocytes were subjected to tachypacing as described before (2,3,9). In short, the spontaneous rate of HL-1 myocytes is ~1 Hz. HL-1 myocytes were subjected to normal electrical field stimulation (1 Hz) for at least 30 min before tachypacing via the C-Pace100™-Culture Pacer (IonOptix Corporation, The Netherlands). Tachypacing was performed at 4 Hz with 20-ms pulses for 8 hours to induce CaT reduction and 1 Hz pacing was used as a control.

Protein-extraction and Western blot analysis

Western-blot analysis was performed as described previously (2,3). Equal amount of protein in SDS-PAGE sample buffer was sonicated before separation on 10% PAA-SDS gels. After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against HSPB1 (SPA801, StressGen USA), V5 tag (Invitrogen, The Netherlands) or GAPDH (Affinity Reagents, The Netherlands). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry.

Live imaging and measurement of CaT

To measure CaT, 2μM of the Ca^{2+}-sensitive Fluo-4-AM dye (Invitrogen, The Netherlands) was loaded into HL-1 myocytes by 45 min incubation, followed by 3 times washing with DMEM solution. Ca^{2+} loaded myocytes were excited by 488 nm and light emitted at 500-550 nm and visually recorded with a 40x-objective, using a Solamere-Nipkow-Confocal-Live-Cell-Imaging system (based on a Leica DM IRE2 Inverted microscope). The live recording of CaT in HL-1 myocytes was performed at 1Hz of stimulation in a temperature (37°C) controlled system. By use of the software ImageJ (National Institutes of Health, USA), the absolute value of fluorescent signals in live myocytes were recorded and analyzed. To compare the fluorescent signals between experiments, the following calibration was utilized: F_{cal}=F/F0, in which (F) is fluorescent dye at any given time and (F0) is fluorescent signal at rest (10). Mean values from each experimental condition were based on 7 consecutive CaT in at least 50 myocytes.

Immunofluorescent staining and confocal analysis

Twenty-four hours after transient transfection of HSPB1, HSPB5, HSPB6, HSPB7 and

HSPB8, HL-1 myocytes were subjected to (tachy)pacing. Afterwards, the myocytes were fixed with 3.7% formaldehyde for 15 minutes, washed three times with Phosphate-Buffered Saline (PBS), permeabilized with 0.2% Triton-X100 and blocked with 0.1 glycine (10 minutes at room temperature) and 5% BSA (30 minutes at room temperature). Antibodies against HSPB6, HSPB7 and HSPB8 (all Abcam, The Netherlands), and HSPB1 and HSPB5 (StressGen, USA) were used as primary antibody. Fluorescein labeled isothiocyanate (FITC) anti-mouse or anti-rabbit (Jackson ImmunoResearch, The Netherlands) were used as secondary antibodies. To visualize F-actin, rhodamine phalloidin (Invitrogen, The Netherlands) was diluted with PBS at 1:40, followed by incubation for 20 minutes at room temperature and washed three times with PBS. Images of FITC and rhodamine fluorescence were obtained using the Leica confocal laser scanning microscope (Leica SP2 AOBS) with 63X/1.4 oil lens. The captured images were processed using Leica Confocal Software and Adobe Photoshop. For determination of the amount of F-actin stress fibers, the intensity of fluorescence was analyzed by ImageJ in 5 independently taken fields. HL-1 myocytes were treated 4 hours prior and during (tachy)pacing with the ROCK inhibitor Y27632 (10μM, Sigma, The Netherlands) to prevent F-actin stress fiber formation. For determination of the amount of colocalization of HSPB with stress fibers, ImagePro software was used. The amount of colocalization was determined as the ratio of total red signal (F-actin) divided by yellow signal (colocalization HSPB with F-actin). Between 400 and 500 myocytes were quantified per condition.

Short interfering RNA of HSPB1 in combination with over-expression of HSPB6, HSPB7 or HSPB8

Downregulation of endogenous HSPB1 was performed as described previously (3). HL-1 myocytes were transiently transfected with HSPB1 siRNA or mock constructs for 5 days. Furthermore, 24 hours before tachypacing, cells were co-transfected with HSPB6,

HSPB7 or HSPB8 construct. After 8-hours (tachy)pacing, CaT were measured and analyzed.

RhoA GTPase activity measurement with G-LISA

For the quantitative analysis of active RhoA GTP levels, G-LISA RhoA Activation Assay (Cytoskeleton, USA) was performed according to the manufacturer protocol. Briefly, 48 hours after the transfection of HL-1 myocytes, myocytes were subjected to (tachy)pacing for 6 hours or directly lysed in lysis buffer and cells were harvested. After measurement of the protein concentration with the use of Precision Red (supplied), equal amounts of lysates were incubated in RhoA GTP affinity plates. The amount of bound RhoA GTP was detected by using primary anti-RhoA antibody (1:250, supplied) and secondary HRP-labeled antibody (1:62.5, supplied). Colorimetric detection at 490nm was performed immediately (BioRad, The Netherlands).

Actin (de-)polymerization-assay

To determine the direct effect of HSPBs on actin polymerization and depolymerization an actin polymerization biochem kit (Cytoskeleton, USA) was used. Twenty-four hours after transient transfection of HL-1 myocytes with HSPB1, HSPB5, HSPB6, HSPB7 or HSPB8, myocytes were lysed in a mild lysis buffer according to the manufacturer protocol. As a control, recombinant human HSPB1 (Stressgen, USA) was dissolved in lysis buffer. Base-line fluorescence of pyrene conjugated actin was measured (Ex. 350 nm; Em. 405 nm) for three minutes, after which cell lysates and recombinant HSPB1 were added to measure effect on (de-)polymerization. Fluorescence was assayed every 60 s for twenty minutes. Maximum actin polymerization was determined by adding polymerization buffer.

Statistical analysis

Results are expressed as mean ± SEM. All CaT measurements were performed in at least triple series. Mean values from each experimental condition were based on 7 consecutive CaT in at least 50 myocytes. ANOVA was used for multiple-group comparisons. All p-values were two-sided. $P<0.05$ was considered statistically significant. SPSS version 16.0 was used for statistical evaluation.

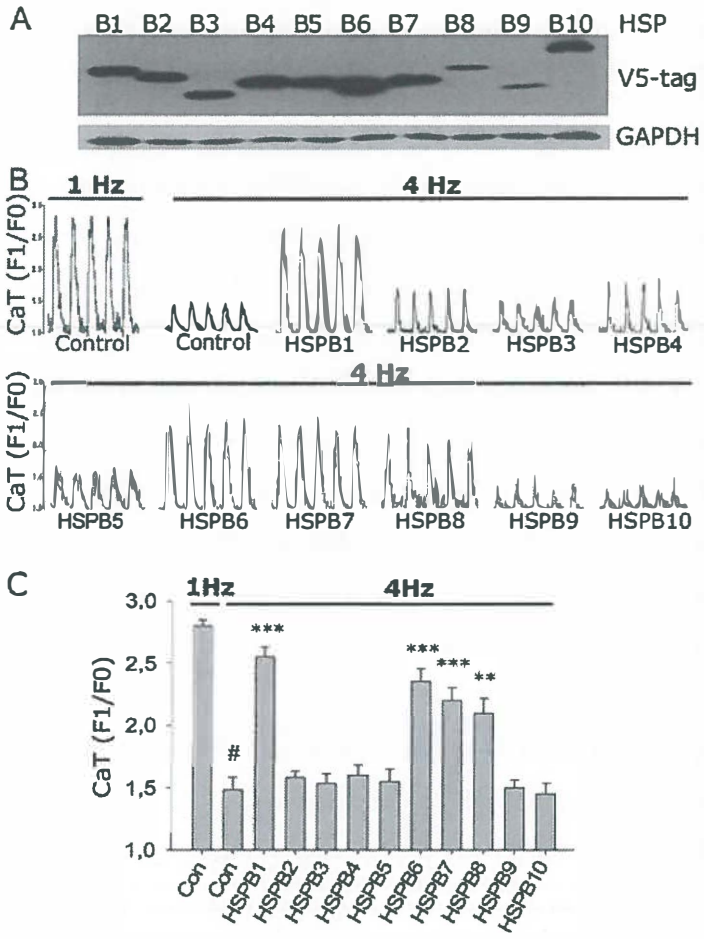


Figure 1 HSPB1, HSPB6, HSPB7 and HSPB8 overexpression prevents against tachypacing-induced CaT reductions in HL-1 myocytes. **(A)** Representative Western blot showing overexpression of HSPB1-10 in transiently transfected HL-1 myocytes. **(B)** Original recordings of CaT in 1 myocyte each from groups indicated. **(C)** Mean CaT data of HSPB1-10 overexpressing myocytes tachypaced (4 Hz) or normal paced cells (1 Hz). $^{**}P<0.01$, $^{***}P<0.001$ vs control tachypaced (4 Hz). # $P<0.001$ vs control normal paced (1 Hz).

Results

Effect of overexpression of the ten different HSPB members on tachypacing-induced remodeling in HL-1 myocytes.

In humans, the HSPB family comprises a group of 10 members with monomeric molecular weight varying between 16 to 28 kDa (5,6,8). Induction of HSPB1 has been shown previously to protect against atrial tachypacing-induced remodeling, including CaT reduction (3). To study the effect of individual HSPB members, HL-1 myocytes were transfected with V5 tagged constructs for each member. All members were successfully overexpressed, albeit HSPB8 and HSPB9 at a lower level (Figure 1A). As a control group, HL-1 myocytes were transfected with an empty vector. None of the overexpressed HSPB members changed CaT in control myocytes paced at 1Hz (data not shown). As observed before (3), tachypacing at 4Hz of HL-1 myocytes resulted in a significant and progressive reduction in CaT (SI Figure S1), which was attenuated by HSPB1 (Figure 1B,C SI movies S1-3). In addition, overexpression of HSPB6, HSPB7 and HSPB8 also protected against tachypacing-induced CaT depression, whereas the other members were ineffective (Figure 1B,C and SI movies S1-12). These results indicate that in addition to HSPB1 also HSPB6, HSPB7 and HSPB8 protect against tachypacing-induced CaT reduction.

HSPB6, HSPB7 and HSPB8 protection against tachypacing-induced CaT reduction is independent of endogenous HSPB1 expression.

HSPB members are known for their ability to form hetero-oligomeric complexes (11,12) and given the fact that HSPB1 is constitutively expressed in HL-1 myocytes, the possibility existed that the protective effect of HSPB6, HSPB7 or HSPB8 on tachypacing-induced CaT reduction was related to (indirect) effects via (oligomerization with) HSPB1. Also, ectopic HSPB expression may induce a stress response in cells leading to the up-regulation of endogenous HSPB1. To exclude these

possibilities, it was first determined whether overexpression of HSPB6, HSPB7 or HSPB8 increased expression of endogenous HSPB1 levels. As shown in Figure 2, endogenous HSPB1 levels were similar after normal pacing (1Hz) and tachypacing (4Hz), irrespective of HSPB6, HSPB7 or HSPB8 overexpression. Secondly, the endogenous HSPB1 level was suppressed by short hairpin RNAs (Figure 3A). In HSPB1 depleted myocytes, HSPB6, HSPB7 or HSPB8 overexpression could still protect against tachypacing-induced CaT reduction (Figure 3B,C, SI movies S13-18). In summary, these results suggest that the protective effects of HSPB6, HSPB7 and HSPB8 against tachypacing-induced CaT reduction are independent of HSPB1.

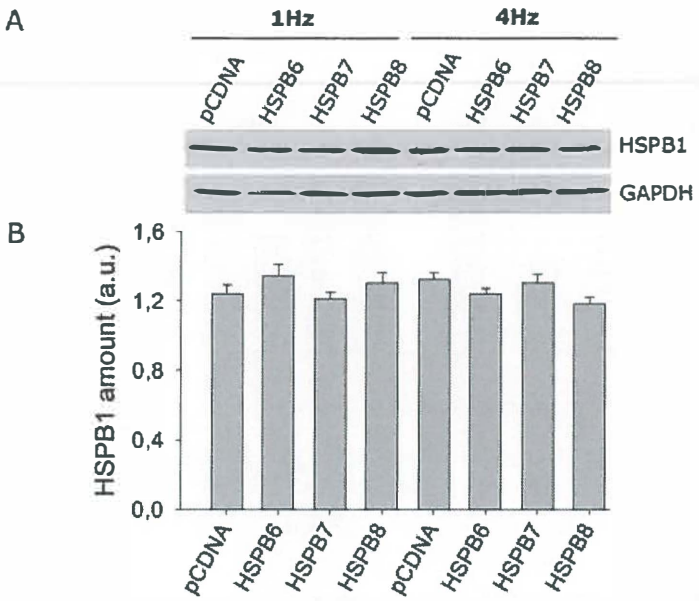


Figure 2 Overexpression of HSPB6, HSPB7 or HSPB8 do not result in changes in endogenous HSPB1 levels. **(A)** Representative Western blot showing that the endogenous HSPB1 levels in transfected HSPB6, HSPB7 and HSPB8 overexpressing HL-1 myocytes are not changed in normal paced myocytes (1 Hz) or tachypaced myocytes (4 Hz). **(B)** Corresponding mean data (n = 3 experiments/group).

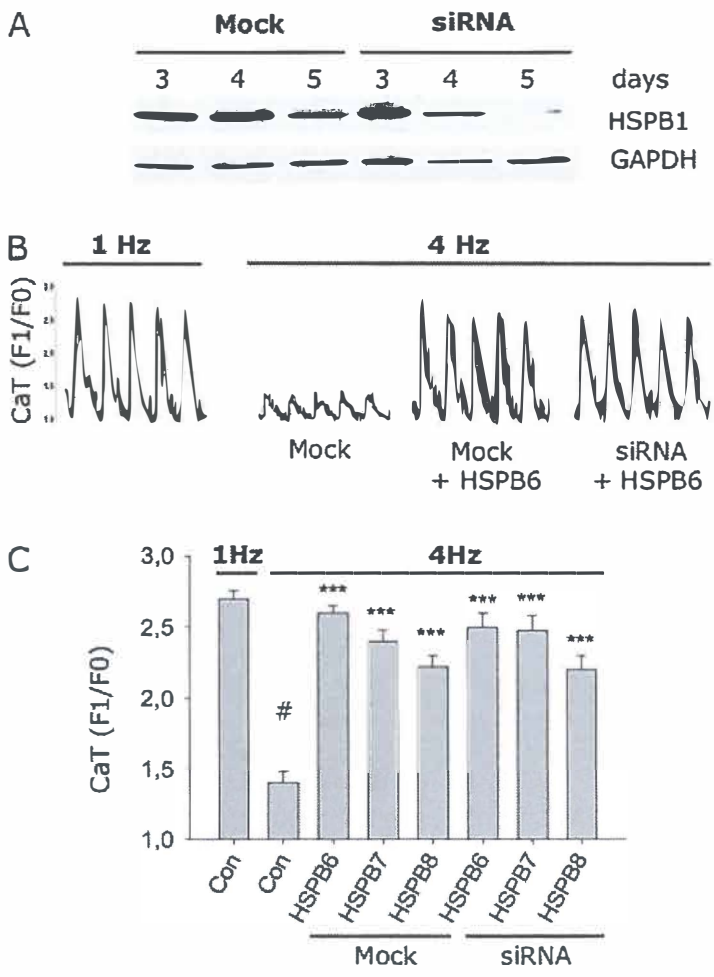


Figure 3 HSPB6, HSPB7, HSPB8 protective effect is independent of HSPB1. **(A)** Western blot showing efficient siRNA-induced HSPB1 knockdown in HL-1 mycoytes. **(B)** Recordings of CaT for mock and siRNA construct. HL-1 myocytes were transfected with mock or siRNA construct for 5 days before study. One day before tachypacing, the myocytes were transfected with HSPB6, HSPB7 or HSPB8 and subjected to normal pacing (1 Hz) or tachypacing (4 Hz). **(C)** Mean CaT data. The cardioprotective effect of HSPB6, HSPB7 or HSPB8 was not blocked by HSPB1 suppression. ***$P<0.001$ vs control tachypaced (4 Hz). # $P<0.001$ vs control normal paced (1 Hz).

HSPB6, HSPB7 and HSPB8 reduce the amount of F-actin stress fibers after tachypacing in HL-1 myocytes.

Calcium signaling is known to be markedly influenced by the stabilization of the cytoskeleton (13-15). F-actin is one of the major components of the cytoskeleton and located under the plasma membrane to maintain cell shape, rigidity and integrity (16,17). Several HSPB members, including HSPB1, HSPB5, HSPB6, HSPB7 and HSPB8, have been reported to be involved in cytoskeletal stability (18-21). To study if the underlying mechanism for HSPB protection is related to effects on actin, immunofluorescent staining was performed. We observed a 1.7 fold induction in the amount of F-actin stress fibers in tachypaced HL-1 myocytes compared to normal paced control myocytes (Figure 4, 5B), an effect that was significantly reduced by overexpression of HSPB1, HSPB6, HSPB7 or HSPB8 (Figure 5A,B). Overexpression of HSPB5, which did not show protection against tachypacing-induced CaT reductions (Figure 1), also did not lead to a reduction in the amount of tachypacing-induced F-actin stress fibers (Figure 4, 5B). Although in tachypaced HSPB1, HSPB6, HSPB7 or HSPB8 overexpressing myocytes a reduction in the amount of F-actin stress fibers was found, HSPB1, HSPB6, HSPB7 and to a lesser extent HSPB8 colocalized with the F-actin residues after tachypacing and this was not the case for HSPB5 (Figure 4, 5A,C). Taken together, these results suggest that HSPB1, HSPB6, HSPB7 and HSPB8 prevent the formation of F-actin stress fibers in tachypaced HL-1 myocytes, and thereby stabilize the cytoskeleton and myocyte function.

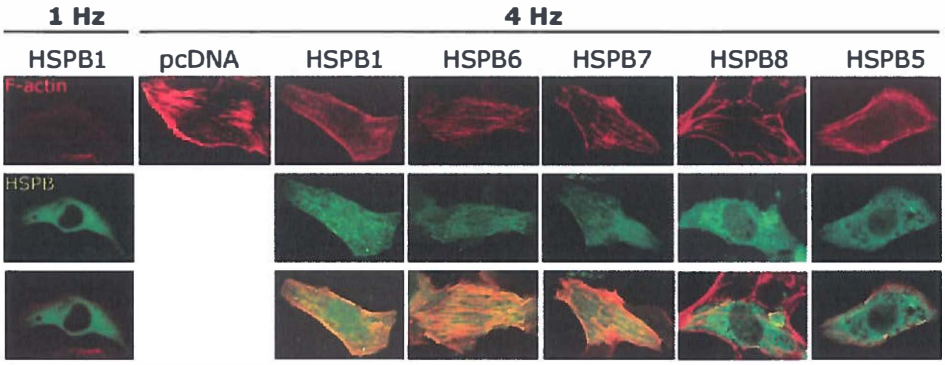


Figure 4 HSPB1, HSPB6 and HSPB7 coocalize with tachypacing-induced F-actin stress fibers in HL-1 myocytes.Immunofluorescent staining of F-actin stress fibers (red) and HSPB positive myocytes (green), in tachypaced HL-1 myocytes (4 Hz). A normal paced (1 Hz) HSPB1 transfected myocyte was shown as a representative control example.

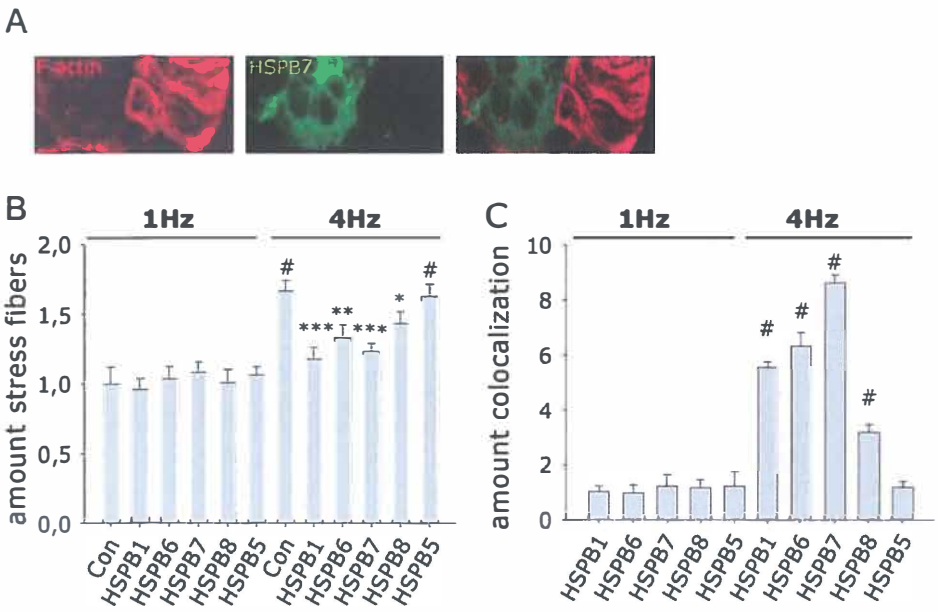


Figure 5 HSPB1, HSPB6, HSPB7 and HSPB8 overexpression is associated with a reduction in the amount of tachypacing-induced F-actin stress fibers in HL-1 myocytes. **(A)** Immunofluorescent staining of F-actin stress fibers (red) and HSPB7 positive myocytes (green), in tachypaced HL-1 myocytes (4 Hz). HSPB7 positive myocytes reveal less stress fibers. **(B)** Quantification of the amount of F-actin stress fibers in HSPB transfected HL-1 myocytes after normal pacing (1 Hz) or

tachypacing (4 Hz). **(C)** Quantification of the amount of colocalization of transfected HSPB with F-actin stress fibers. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control tachypaced myocytes (4 Hz), #*P*<0.05 vs control normal paced (1 Hz).

Tachypacing induces RhoA GTPase and ROCK activation, resulting in F-actin formation and reduction in calcium transients.

To confirm the role of RhoA GTPase pathway in tachypacing-induced F-actin stress fiber formation and reductions in CaT, HL-1 myocytes were tachypaced for 0-8 hours and RhoA GTPase activity was measured in cell lysates (Figure 6A). A significant induction of the RhoA GTPase activity was observed at 6 hours of tachypacing. In parallel, the amount of F-actin was quantified. A gradual increase in the amount of F-actin was observed during tachypacing, which was prevented by the ROCK inhibitor Y27632 (10μM) (Figure 6B). In addition, also tachypacing-induced changes in CaT were reduced by Y27632 (Figure 6C), indicating that also this effect is RhoA-mediated.

HSPB8, but not HSPB1, HSPB6, and HSPB7, reduces activation of RhoA GTPases after tachypacing

To test whether the protective effect of HSPB members is related to a direct modulation of the RhoA GTPase activity, RhoA GTPase activity was measured in normal paced (1Hz) and tachypaced (4Hz) HL-1 myocytes transfected with the individual HSPB members. None of the HSPB members affected RhoA GTPase activity in 1Hz paced HL-1 myocytes (SI Figure S2). Only HSPB8 transfected HL-1 myocytes revealed significantly reduced activation of RhoA GTPase upon 6 hours of tachypacing and all other (protective) HSPB members were ineffective (Figure 7), suggesting that their protective effects against tachycardia remodeling are downstream of RhoA GTPase activation.

HSPB1, HSPB6, and HSPB7 prevent G-to-F actin polymerization.

To investigate whether HSPB1, HSPB6, and HSPB7, rather than affecting RhoA GTPase activation, may ameliorate the downstream consequences of activated RhoA GTPase, we

measured their effect on the polymerization of G-actin to F-actin and also the depolymerization, using an *in vitro* polymerization kit. Base-line fluorescence of G/F-actin ratios were measured for three minutes, after which cell lysates from HL-1 myocytes transfected with the respective HSPB members or recombinant HSPB1 were added (Figure 8). The non-protective HSPB5 was used as a control. When polymerization buffer was added to the baseline G/F-actin, a rapid increase in the conversion of G-to-F actin ratio was observed, indicative of fast actin polymerization. Addition of lysates from HSPB1 transfected cells as well the addition of 0.5μg recombinant human HSPB1 induced depolymerization of F-actin. Although less effective, lysates from HSPB6 also induce depolymerization, whereas lysates from HSPB7 transfected myocytes prevent actin polymerization but did not show an effect on depolymerization. In contrast to the findings of HSPB1, HSPB6 and HSPB7 in preventing the formation and/or stimulating the depolymerization of F-actin stress fibers, addition of lysates from HSPB8 transfected cells resulted in actin polymerization, although the levels of polymerization were reduced compared to lysates of the non-protective HSPB5 transfected myocytes, which showed near to normal polymerization.

These results together suggest that HSPB1, HSPB6 and HSPB7 may prevent tachycardia remodeling by directly preventing the formation and/or stimulating the depolymerization of F-actin stress fibers downstream of active RhoA GTPase, whilst HSPB8 mainly acts at the level of tachypacing-induced RhoA GTPase activation.

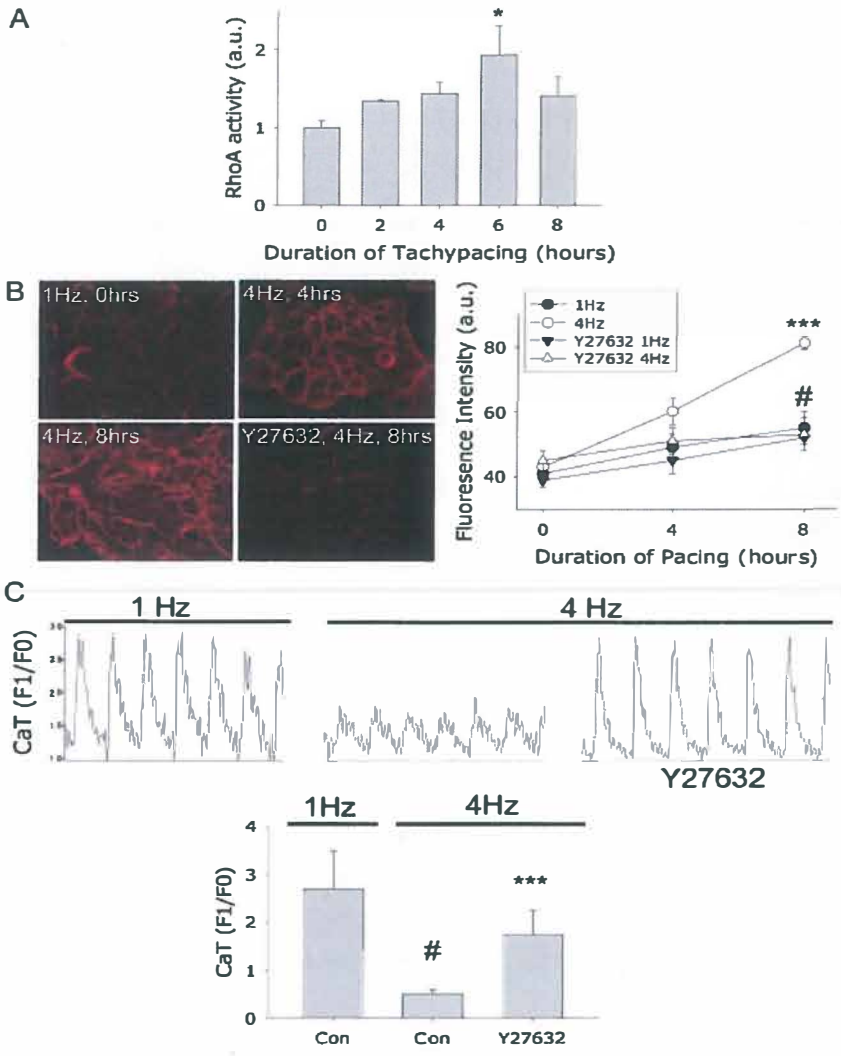


Figure 6 Tachypacing induces gradual activation of RhoA-GTPase and consequently formation of F-actin stress fibers and reduction in CaT in HL-1 myocytes. **(A)** HL-1 myocytes were tachypaced upto 8 hours and activation of RhoA-GTPase was measured. **(B)** Left: Examples of immunofluorescent staining of F-actin stress fibers (red) in tachypaced HL-1 myocytes (4 Hz, 4 and 8 hours), normal paced (1 Hz) and tachypaced HL-1 myocytes pretreated with the ROCK inhibitor Y27632 (4 Hz, 8 hours). Right: quantification of the fluorescence intensity of F-actin in the conditions as indicated. **(C)** Top: Original recordings of CaT in 1 myocyte each from groups indicated. Below: Mean CaT data of normal (1 Hz) and tachypaced (4 Hz) HL-1 myocytes and tachypaced myocytes pretreated with Y27632. ***P<0.001 Y27632 tachypaced vs control tachypaced (4 Hz), #P<0.001 vs control normal paced (1 Hz).

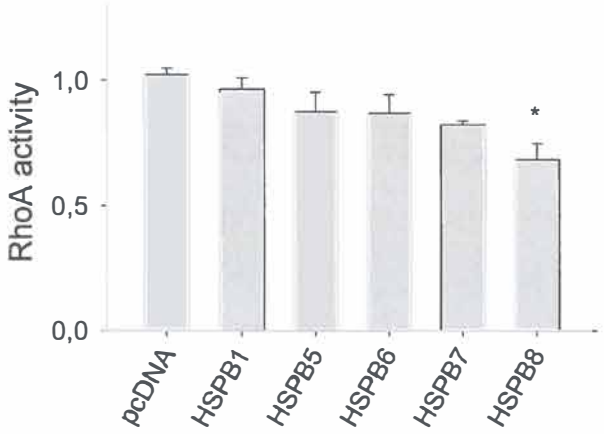


Figure 7 HSPB8 reduces activation of RhoA-GTPase during tachypacing in HL-1 myocytes. HL-1 myocytes were transfected with HSPB1, HSPB5, HSPB6, HSPB7, HSPB8, or empty plasmid (pcDNA) and subjected to tachypacing (4 Hz, 6 hours). Activation of RhoA-GTPase in tachypaced (4 Hz) HL-1 myocytes, transfected with plasmids as indicated, is shown. *P<0.05 vs control tachypaced myocytes (4 Hz).

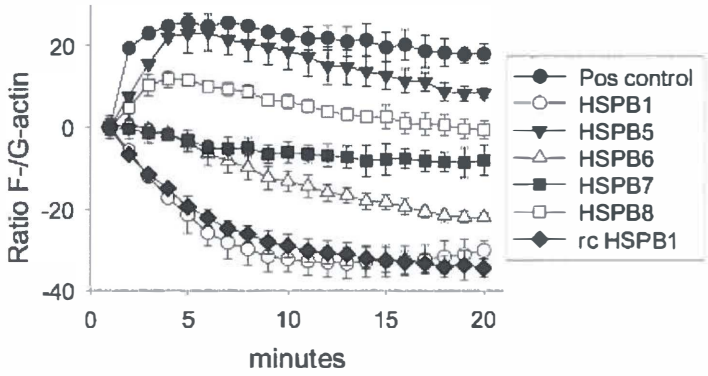


Figure 8 HSPB1, HSPB6, and HSPB7 attentuate F-actin stress fiber formation *in vitro*. Cell-lysates from HL-1 myocytes transfected with HSPB1, HSPB5, HSPB6, HSPB7, or HSPB8 and recombinant human HSPB1 were used to measure influence of HSPB members on polymerization of G-actin to F-actin and also the depolymerization. ** P<0.01 vs lysates of HSPB5 transfected myocytes at 20 minutes incubation, *** P<0.001 vs lysates of HSPB5 transfected myocytes at 20 minutes incubation.

Discussion

Previously, we showed HSPB1 to protect in HL-1 myocytes against tachycardia remodeling and to preserve normal Ca^{2+} transients as well as the actin cytoskeleton upon tachypacing (2,3). In the current study we found that, in addition to HSPB1, also some other members of the HSPB family (HSPB6, HSPB7 and HSPB8) display protective effects against tachypacing-induced remodeling. Interestingly, all protective HSPB members reduced the formation of F-actin stress fibers, although their modes of action differ. Whereas HSPB8 interfered with tachypacing-induced RhoA GTPase activity, HSPB1, HSPB6, and HSPB7 did not. HSPB1, HSPB6 and HSPB7 were found to directly inhibit G- to F-actin polymerization and/or stimulate depolymerization, indicating a protective role against tachycardia remodeling downstream of RhoA GTPase activation.

Role of Rho GTPases in induction of AF

The current study revealed a prime role for tachypacing-induced RhoA GTPase activity and consequently F-actin stress fiber formation in reductions in calcium transients. This finding is in line with studies revealing an important role for Rho GTPases, including RhoA and Rac1, in formation of F-actin stress fibers (22) and the initiation of AF (23,24). Consistently, experimental studies showed that activation of RhoA GTPases result in conduction disturbances and cardiac dysfunction similar to those described in AF (25,26). Rho GTPases represent a family of small GTP-binding proteins involved in cell cytoskeleton organization, migration, transcription, and proliferation. Rho GTPases have gained considerable recognition as powerful regulators of actin cytoskeletal organization in the heart (22). It was observed that active Rho GTPases stimulate the conversion of G-actin to F-actin, which results in cytoskeletal injury including changes in calcium signaling, conduction disturbances and contractile dysfunction (13-16), which are all substrates for the development of AF (25,26). Actin stress fiber assembly and contraction are predominantly mediated by Rho-associated serine/threonine kinase (ROCK), a major

down-stream effector of the Rho pathway. Consistent with all of the above, we now show that tachypacing activates RhoA and that inhibition of ROCK, its effector of actin polymerization, prevents tachypacing-induced reductions in CaT.

The HSPB family

Whereas all HSPB members are characterized by the presence of a conserved crystallin domain, this domain is flanked by N- and C-termini that shows large sequence divergence between the members (Table 1) (5,8). Also, the four members (HSPB1, HSPB6, HSPB7 and HSPB8) that we found to have protective effects against tachycardia remodeling show, besides sequence divergence, a number of structural and functional differences (see below). Interestingly, however, all four members, together with the non-protective HSPB5, show high basal expression in heart tissue (Table 1). In addition, three members (HSPB1, HSPB6, HSPB7) seemed to act similar in AF protection, i.e. preventing actin remodeling downstream of RhoA-activation. Only HSPB8 appears to directly affect RhoA-activation. So, the question is what are the characteristics shared by these members and, in addition, what are the differences between them that can explain their protective effects on tachypacing-induced remodeling?

In cell-free assays, small HSPs have been shown to act as ATP-independent "holdases", maintaining unfolded or misfolded proteins in a folding competent, non-aggregated state, hereby supporting refolding by ATP-regulated chaperones, in particular the HSP70 machinery (8). In cellular assays, however, of the four cardioprotective HSPB members, only HSPB1 seems to support such refolding reaction (27,28). Moreover, HSPB5 also shares this activity (28), but did not reveal protective effects against tachycardia remodeling. This finding indicates that such a chaperone-like activity is not of prime importance to the HSPB-mediated protective effects as reported in the current study.

Several members of the HSPB family, including HSPB6, HSPB7 and HSPB8 were recently shown to be able to assist in the clearance of stress-induced misfolded proteins,

in part through interaction with (HSPB7) or activation of (HSPB8) the macro-autophagy machinery (28-30). Yet, this activity is not shared by e.g. HSPB1 whilst HSPB9 that also can enhance clearance of misfolded proteins (28), albeit likely via proteasomal degradation (31) had no effect on AF. So, the clearance of misfolded proteins seems not to be a common target of all cardioprotective HSP members.

Another feature shared amongst many HSPB members is their dynamic (de)oligomerisation (5,8). This characteristic has been suggested to be crucial for e.g. the ability of HSPB1 to interact with several cytoskeletal components, including actin, intermediate filaments, and microtubules (7,32). Yet, in cells HSPB7 and HSPB8 do not appear to be present in large oligomeric structures (31,33) implying also that this does not edify their protective role against tachycardia remodeling. However, all protective HSPB members can be found in cells as non-oligomeric (most likely dimeric) proteins as well. For HSPB1, dimers have been suggested to be the active species in regulating actin (re)polymerization after stress (34). Also for HSPB6 stress-induced translocation to actin of the myofibrils has been reported, which has been associated with improved heart function (32). Also HSPB7 translocates from cytosol to the Z-/I-area of myofibrils, and thereby exerts a protective effect to ischemic stress (21). This interaction may be mediated via α-filamin, an actin-binding protein (35). All of this is consistent with our current findings that HSPB1, HSPB6 and HSPB7 are associated with F-actin stress fibers upon tachypacing and the fact that they can directly prevent actin polymerization, an effect that occurs in living cells as a down-stream effect of Rho activation. In addition, the findings also suggest that chaperone-like (refolding or clearance) function and actin protection are distinct, uncoupled functions of these HSPB members. For HSPB8, the remaining AF protecting HSPB member, no direct association with actin and/or microtubules has been reported so far. Although HSPB8 is highly expressed in heart and muscle and anti-HSPB8 antibodies decorate sarcomeres (30), only a weak association with F-actin stress fibers after tachypacing was observed in the current study. Moreover,

we only found weak attenuating effects of HSPB8 on actin polymerization. So, the protective effects of HSPB8 against tachycardia remodeling seem distinct from that of the other HSPB members. Consistently, we indeed observed that HSPB8 was the only member that directly affected tachypacing-induced RhoA activation. How HSPB8 may modulate this effect remains an enigma, but maybe its unique role within the HSPB family in activating autophagy (28,29) may be important. Autophagy may prevent protein aggregate formation that served as an early trigger for RhoA activation. Indeed, preventing aggregate formation has been suggested as the mode by which HSPB8 can prevents desmin-related cardiomyopathy (36).

The present study demonstrates that RhoA activation plays a central role in tachypacing-induced myocyte remodeling. This remodeling can be prevented by some, but not all, members of the HSPB family. This protection is not directly related to canonical chaperone-like function of these HSPB members, but involves prevention of RhoA activation (HSPB8) or its downstream action on actin remodeling (HSPB1, HSPB6, HSPB7). The findings widen the possibilities for the identification of novel therapeutic approaches directed at RhoA activating components or boosting the expression of one or more of the cardioprotective HSPB members.

Supporting Information

It can be accessed online at:
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0020395

Table 1: characteristics of small HSPB members (37,38)

Gene Name	Protein Name	Alternative Name	Sequence Identity	Molecular Size (kDa)	Heat Inducibility	Expression in Heart	Other tissue expression
HSPB1	HSPB1	Hsp25, HSP27, HSP28	100%	22.783	Yes	+++	Uterus, skin, platelets, brain, kidney, some tumor cells
HSPB2	HSPB2	MKBP	36%	20.233	No	+	Skeletal muscle
HSPB3	HSPB3	HSPL27	23%	16.966		+	Skeletal muscle
HSPB4	HSPB4	αA-crystallin, CRYAA, CRYA1	36%	19.909	No	-	Lens of eye, spleen
HSPB5	HSPB5	αB-crystallin, CRYAB, CRYA2	38%	20.159	Yes	++++	Lens of eye, vascular wall cells, lung, kidney, brain, some tumor cells
HSPB6	HSPB6	Hsp20, p20	34%	17.136	No	++	Skeletal muscle, stomach, liver, lung, kidney, platelet
HSPB7	HSPB7	cvHsp	20%	18.611	?	+++++	Skeletal muscle
HSPB8	HSPB8	Hsp22, H11	34%	21.604	Yes	++	Skeletal muscle, stomach, liver, lung, kidney, brain
HSPB9	HSPB9	FLJ27437	19%	17.486	?	-	Testis
HSPB10	HSPB10	ODF1	17%	28.366	?	-	Testis

doi:10.1371/journal.pone.0020395.t001

References

1. Dobrev D and Nattel S (2010) New antiarrhythmic drugs for treatment of atrial fibrillation. *Lancet*, **375**, 1212-1223.

2. Brundel BJJM, Henning RH, Ke L, Van Gelder IC, Crijns HJGM, et al. (2006) Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation. *J.Mol.Cell Cardiol.*, **41**, 555-562.

3. Brundel BJJM, Shiroshita-Takeshita A, Qi XY, Yeh YH, Chartier D, et al. (2006) Induction of heat-shock response protects the heart against atrial fibrillation. *Circ Res*, **99**, 1394-1402.

4. Morimoto RI (2008) Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev*, **22**, 1427-1438.

5. Kappé G, Franck E, Verschuure P, Boelens WC, Leunissen JA, et al. (2003) The human genome encodes 10 alpha-crystallin-related small heat shock proteins: HspB1-10. *Cell Stress Chaperones.*, **8**, 53-61.

6. Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, et al. (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones.*, **14**, 105-111.

7. Landry J and Huot J (1995) Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem Cell Biol*, **73**, 703-707.

8. Vos MJ, Hageman J, Carra S, and Kampinga HH (2008) Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. *Biochemistry*, **47**, 7001-7011.

9. Brundel BJJM, Kampinga HH, and Henning RH (2004) Calpain Inhibition Prevents Pacing induced Cellular Remodeling in a HL-1 Myocyte Model for Atrial Fibrillation. *Cardiovasc Res*, **62**, 521-528.

10. Paredes RM, Etzler JC, Watts LT, Zheng W, and Lechleiter JD (2008) Chemical calcium indicators. *Methods*, **46**, 143-151.

11. Sun XS, Fontaine JM, Rest JS, Shelden EA, Welsh MJ, et al. (2004) Interaction of human hsp22 (HSPB8) with other small heat shock proteins. *J.Biol.Chem.*, **279**, 2394-2402.

12. Simon S, Fontaine JM, Martin JL, Sun X, Hoppe AD, et al. (2007) Myopathy-associated alphaB-crystallin mutants: abnormal phosphorylation, intracellular location, and interactions with other small heat shock proteins. *J.Biol.Chem.*, **282**, 34276-34287.

13. Johnson BD, Scheuer T, and Catterall WA (2005) Convergent regulation of skeletal muscle Ca2+ channels by dystrophin, the actin cytoskeleton, and cAMP-dependent protein kinase. *Proc Natl Acad Sci*, **102**, 4191-4196.

14. Leach RN, Desai JC, and Orchard CH (2005) Effect of cytoskeleton disruptors on L-type Ca channel

distribution in rat ventricular myocytes. *Cell Calcium*, **38**, 515-526.

15. Kerfant BG, Vassort G, and Gomez AM (2001) Microtubule disruption by colchicine reversibly enhances calcium signaling in intact rat cardiac myocytes. *Circ Res.*, **88**, E59-E65.

16. Pollard TD and Cooper JA (2009) Actin, a central player in cell shape and movement. *Science*, **326**, 1208-1212.

17. Janmey PA (1998) The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev.*, **78**, 763-781.

18. Hoch B, Lutsch G, Schlegel WP, Stahl J, Wallukat G, et al. (1996) HSP25 in isolated perfused rat hearts: localization and response to hyperthermia. *Mol Cell Biochem*, **160-161**, 231-239.

19. Fan GC, Chu G, Mitton B, Song Q, Yuan Q, et al. (2004) Small heat-shock protein HSP20 phosphorylation inhibits beta-agonist-induced cardiac apoptosis. *Circ Res*, **94**, 1474-1482.

20. Sui X, Li D, Qiu H, Gaussin V, and Depre C (2009) Activation of the bone morphogenetic protein receptor by H11kinase/Hsp22 promotes cardiac cell growth and survival. *Circ Res*, **104**, 887-895.

21. Golenhofen N, Perng MD, Quinlan RA, and Drenckhahn D (2004) Comparison of the small heat shock proteins alphaB-crystallin, MKBP, HSP25, HSP20, and cvHSP in heart and skeletal muscle. *Histochem Cell Biol.*, **122**, 415-425.

22. Brown JH, Del Re DP, and Sussman MA (2006) The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res*, **98**, 730-742.

23. Sah VP, Minamisawa S, Tam SP, Wu TH, Dorn GW, et al. (1999) Cardiac-specific overexpression of RhoA results in sinus and atrioventricular nodal dysfunction and contractile failure. *J Clin Invest*, **103**, 1627-1634.

24. Adam O, Frost G, Custodis F, Sussman MA, Schäfers HJ, et al. (2007) Role of Rac1 GTPase activation in atrial fibrillation. *J Am Coll Cardiol.*, **50**, 367.

25. Reil JC, Hohl M, Oberhofer M, Kazakov A, Kaestner L, et al. (2010) Cardiac Rac1 overexpression in mice creates a substrate for atrial arrhythmias characterized by structural remodelling. *Cardiovasc Res*, **87**, 485-493.

26. Ogata T, Ueyama T, Isodono K, Tagawa M, Takehara N, et al. (2008) MURC, a muscle-restricted coiled-coil protein that modulates the Rho/ROCK pathway, induces cardiac dysfunction and conduction disturbance. *Mol Cell Biol.*, **28**, 3424-3436.

27. Bryantsev AL, Loktionova SA, Ilyinskaya OP, Tararak EM, Kampinga HH, et al. (2002) Distribution, phosphorylation, and activities of Hsp25 in heat-stressed H9c2 myoblasts: a functional link to cytoprotection.

Cell Stress Chaperones, **7**, 146-155.

28. Vos MJ, Zijlstra MP, Kanon B, Van Waarde-Verhagen MA, Brunt ER, et al. (2010) HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones. *Hum Mol Genet.*, **19**, 4677-4693.

29. Carra S, Brunsting JF, Lambert H, Landry J, and Kampinga HH (2009) HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2{alpha} phosphorylation. *J Biol Chem*, **284**, 5523-5532.

30. Carra S, Boncoraglio A, Kanon B, Brunsting JF, Minoia M, et al. (2010) Identification of the Drosophila ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J.Biol.Chem.*, **285**, 37811-37822.

31. Vos MJ, Zijlstra MP, Carra S, Sibon OC, and Kampinga HH (2010) Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy*, **7**, 1-3.

32. Fan GC and Kranias EG (2010) Small heat shock protein 20 (HspB6) in cardiac hypertrophy and failure. *J Mol Cell Cardiol*, **in press**.

33. Carra S, Seguin SJ, Lambert H, and Landry J (2008) HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem*, **283**, 1437-1444.

34. Lavoie JN, Lambert H, Hickey E, Weber LA , and Landry J (1995) Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol*, **15**, 505-516.

35. Krief S, Faivre JF, Robert P, Le Douarin B, Brument-Larignon N, et al. (1999) Identification and characterization of cvHsp. A novel human small stress protein selectively expressed in cardiovascular and insulin-sensitive tissues. *J Biol Chem.*, **274**, 36592-36600.

36. Sanbe A, Daicho T, Mizutani R, Endo T, Miyauchi N, et al. (2009) Protective effect of geranylgeranylacetone via enhancement of HSPB8 induction in desmin-related cardiomyopathy. *PlosOne*, **4**, e5351.

37. Vos MJ, Zijlstra MP, Carra S, Sibon OC, and Kampinga HH (2011) Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy*, **7**, 101-103.

38. Vos MJ (2009) Small heat shock proteins; implications for neurodegeneration and longevity. *Thesis*.

CHAPTER 5

Effects of different small HSPB members on contractile dysfunction and structural changes in a *Drosophila melanogaster* model for Atrial Fibrillation

Deli Zhang [a*], Lei Ke [b*], Katarina Mackovicova [a], Johannes J.L. Van Der Want [c], Ody C.M. Sibon [b], Robert M. Tanguay [d], Genevieve Morrow [d], Robert H. Henning [a], Harm H. Kampinga [b], Bianca J.J.M. Brundel [a]

[a] Department of Clinical Pharmacology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands.
[b] Department of Cell Biology, section Radiation and Stress Cell Biology, University Institute for Drug Exploration (GUIDE), University of Groningen, University Medical Center Groningen, The Netherlands.
[c] Department of Cell Biology, section Molecular Imaging and Electron Microscopy, University of Groningen, University Medical Center Groningen, The Netherlands.
[d] Laboratory of Cell and Developmental Genetics, Department of Molecular Biology, Medical Biochemistry and Pathology, Institut de Biologie Intégrative et des Systèmes and PROTEO, Université Laval, Québec, Canada.
* Both authors contributed equally to this work.

J Mol Cell Cardiol. 2011; 51(3): 381-389

Abstract

The most common clinical tachycardia, Atrial Fibrillation (AF), is a progressive disease, caused by cardiomyocyte remodeling, which finally results in contractile dysfunction and AF persistence. Recently, we identified a protective role of heat shock proteins (HSPs), especially the small HSPB1 member, against tachycardia remodeling in experimental AF models. Our understanding of tachycardia remodeling and anti-remodeling drugs is currently hampered by the lack of suitable (genetic) manipulatable *in vivo* models for rapid screening of key targets in remodeling. We hypothesized that *Drosophila melanogaster* can be exploited to study tachycardia remodeling and protective effects of HSPs by drug treatments or by utilizing genetically manipulated small HSP-overexpressing strains. Tachypacing of *Drosophila* pupae resulted in gradual and significant cardiomyocyte remodeling, demonstrated by reduced contraction rate, increase in arrhythmic episodes and reduction in heart wall shortening, compared to normal paced pupae. Heat shock, or pre-treatment with HSP-inducers GGA and BGP-15, resulted in endogenous HSP overexpression and protection against tachycardia remodeling. DmHSP23 overexpressing *Drosophilas* were protected against tachycardia remodeling, in contrast to overexpression of other small HSPs (DmHSP27, DmHSP67Bc, DmCG4461, DmCG7409, DmCG14207). (Ultra)structural evaluation of the tachypaced heart wall revealed loss of sarcomeres and mitochondrial damage which were absent in tachypaced DmHSP23 overexpressing *Drosophila*. In addition, tachypacing induced a significant increase in calpain activity, which was prevented in tachypaced *Drosophilas* overexpressing DmHSP23. Tachypacing of *Drosophila* resulted in cardiomyocyte remodeling, which was prevented by general HSP-inducing treatments and overexpression of a single small HSP, DmHSP23. Thus, tachypaced *Drosophila melanogaster* can be used as an *in vivo* model system for rapid identification of novel targets to combat AF associated cardiomyocyte remodeling.

Introduction

Atrial Fibrillation (AF) is the most common tachycardia in the clinical setting and it affects patients' cardiovascular function in a progressive and sustained manner (1). AF is characterized by specific changes in electrical, structural and contractile function of the atrial cardiomyocytes, commonly denoted as 'remodeling'. Tachycardia remodeling underlies contractile dysfunction and the progressive and intractable nature of AF (2). Hence, there is great interest in developing anti-remodeling therapies directed at the targets underlying remodeling (3). However, our understanding of tachycardia remodeling that contributes to AF progression and the effects of anti-remodeling drugs is currently hampered by the lack of suitable genetically manipulatable *in vivo* models. There are some *in vitro* cardiomyocyte cell models, using isolated atrial cardiomyocytes or the HL-1 atrial cardiomyocyte cell line (4,5), that can be used for such purposes and that yielded initial suggestions regarding the protective effects of heat shock proteins (HSPs) (4,6,7). Whereas some of the concepts generated in these *in vitro* models could be confirmed in experimental canine models for AF (4,8), the precise translation to the *in vivo* situation is hindered due to limited possibilities for genetic manipulations in experimental models (9) and larger animal models that beside canine models (10), include AF models in goat (11) and sheep (12). Although these animal models have been very useful in obtaining knowledge about concepts of electrical remodeling and contractile dysfunction, they lack the flexibility of dissecting the underlying molecular mechanisms and employing genetic or compound screens. In addition, experimental execution is extensive and expensive. Therefore, we utilize *Drosophila melanogaster* as an *in vivo* model system for tachycardia remodeling, since it has been recognized that *Drosophila* contains powerful genetics and provide tools to manipulate gene expression in a highly precise spatial and temporal fashion, by the use of a UAS/GAL4 system (13,14). Furthermore, 85% of the *Drosophila* genes have human homologues (13,15,16), including several genes that have been associated with human cardiac diseases, including

heart failure, arrhythmias and dilated cardiomyopathy (14,15,17-20). Moreover *Drosophila* contains a pumping heart (13-16). In the current study we report on the development of a tachycardia model in *Drosophila*. In addition, we show, consistent with our previous findings in *in vitro* cardiomyocytes (4,6) and *in vivo* canine models for AF (4,8), that induction of HSPs protects against tachypacing-induced contractile dysfunction. Furthermore, by using both functional and (ultra)structural analyses, it was found that this protection is accomplished upon overexpression of a single HSP, DmHSP23, the possible ortholog of human HSPB1. Moreover, overexpression of the other small DmHSP members did not result in a protective effect. Thus our study demonstrates the feasibility and power of the tachypaced *Drosophila melanogaster* as a model in translational research in the field of tachycardia induced heart failure.

Materials and Methods

Maintenance of *Drosophila melanogaster* strains and HSP-inducing treatments

Drosophila stocks were maintained at 21°C, during experiments at 25°C, according to standard protocols. The W1118 line was used as a control and obtained from Genetic Services Inc. (Massachusetts, USA). Actin-GAL4 (stock #4414) driver strains were obtained from the Bloomington Stock Centre (Indiana University, USA). UAS-DmHSP23 and UAS-DmHSP27 strains were generated from W1118 genetic background and have been described before (21-23). These UAS strains were crossed, using standard genetics, with the actin-GAL4 expressing strains in order to generate strains that contain actin-GAL4 and either UAS-DmHSP23 or UAS-DmHSP27 on the same chromosome. Stable ubiquitous overexpression of DmHSP23 and DmHSP27 was confirmed by Western blotting with specific antibodies. Other *Drosophila* small HSP overexpressing strains included were HSP67Bc, CG4461, CG7409, CG14207 (24,25). These transgenic strains were generated at Genetic Services Inc. by injection of the pUAS vector containing a small HSP of interest with a V5 tag, into the W1118 genetic background. These transgenic strains were crossed with actin-GAL4 expressing flies in order to induce ubiquitous transgenic gene expression of the small DmHSP of interest in the F1 progeny, which was confirmed by Western blotting.

Heat treatment was performed at 37°C for 1 hour followed by an overnight recovery at 25°C. The HSP-inducing compounds Geranylgeranylacetone (GGA, 1mM) and O-(3-piperidino-2-hydroxy-1-propyl)-nicotinic acid amidoxim di-hydrochloride (BGP-15, 1mM) (both kind gifts from Eisai Japan and NP-gene USA, respectively) were freshly dissolved in de-mineralized water and 0.5 ml was added to standard *Drosophila* food for at least 48 hours before (tachy)pacing. Controls were subjected to de-mineralized water only.

Tachypacing of *Drosophila* and measurement of heart function parameters

Early pupae were selected as described before (26). In short, transparent pupae were selected at entry of the immobile phase (this phase continues for about 3 hours) and were placed on 1% agarose gel in PBS. The basal heart rate in the early pupae was about 2.2Hz (Supplementary information Figure 1A). Pupae were tachypaced by using the C-Pace100™-Culture Pacer (IonOptix Corporation, The Netherlands) for 0-120 minutes. Controls were subjected to normal electrical field stimulation similar to basal heart rate (2.2Hz) whereas tachypacing was conducted at 2.3 fold basal rate or as indicated. The heart wall of pupae could follow the pacing rate (Supplementary information Figure 1B). During interruption of (tachy)pacing for about 40 seconds, spontaneous heart function at each time point was visually scored under the light microscope in duplicate periods of 20 seconds. In short, the rate of contraction of the heart was quantified by focussing on the heart wall contrast edges (26) and only regular contractions were included. During (tachy)pacing interruption, also time lapse movies were made and analyzed by edge detection software (Leica Microsystems, Mannheim, Germany) to determine the heart wall shortening, which indicates the strength of contraction (amplitude of diastolic and systolic heart wall contraction), and duration of arrhythmic periods (ImageJ). Arrhythmicity index was calculated as the duration of arrhythmic periods divided by the total duration of measured periods.

Quantitative PCR

From at least five early pupae per condition, total RNA was isolated by utilizing the Nucleospin RNA/protein mini kit (Macherey-Nagel, The Netherlands). First strand cDNA was generated using M-MLV reverse transcriptase (Invitrogen, The Netherlands) using oligo(dT)18 primers (Biologio, The Netherlands). Relative changes in transcription level were determined using the CFX384 Real-Time System C1000 Thermocycler (BioRad, The Netherlands) in combination with SYBR green supermix (Bio-Rad, The Netherlands).

Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems). Fold induction was adjusted using RpL32 transcript levels as a standard. Primer pairs used included the heat inducible DmHSP27 F: CTAGACAGGGTTGTGAATAAAGAG and R: AAACCGAAGTCATCCTCCAG and DmHSP70AA F: ACCTCAACCTATCCATCAACC and R: GTCTCAATTCCC AATGAAAGTG and for RpL32 F: CGATCTCGCCGCAGTAAA and R: GCACCAAGCACTTCATCC. The PCR efficiencies for all primer pairs were between 85% and 100%.

Western blot analysis

Drosophila pupae or adult flies (five per condition) were quickly lysed on ice in 50 µl SDS sample loading buffer (10% SDS, 50% glycerol, 0.33M Tris-HCl pH 6.8, 10% β-mercaptoethanol, 0.05% bromophenol blue) followed by sonification. After a short spin-down, supernatant was collected and boiled for 6 minutes. Proteins were separated on SDS-PAGE 4-20% Precise™ Protein gels (Thermo Scientific) and transferred onto nitrocellulose membranes (GE Healthcare, The Netherlands). The membranes were blocked in 5% skim milk (1 hour, room temperature) and incubated overnight at 4°C with primary *Drosophila* anti-DmHSP23, anti-DmHSP27 antibodies (27,28), anti-V5 antibodies (Invitrogen, The Netherlands) or anti α-tubulin antibodies (Sigma, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by Super Signal (Thermo Scientific, The Netherlands) and quantified by densitometry.

(Ultra) structural evaluation by Light and Electron Microscopy

For morphological evaluation, the head and the abdomen were dissected from the early pupae and the middle segment was immediately fixed for at least 2 hours at 4^0C in 2% glutaraldehyde (in 0.1M cacodylate buffer, pH7.4). Post-fixation was performed for 2

hours in 1% osmium tetroxide (supplemented with 1.5% $K_4Fe(CN)_6$ in cacodylate buffer, pH7.4) at 4^0C. After dehydration in ethanol, pupae were embedded in Epon and semi-thin sections (1μm) were cut and stained with 1% toluidine blue and used for light microscopic evaluation. To verify the ultrastructural changes, ultrathin sections (60nm) were made and stained with uranylacetate and lead citrate and examined in a Philips CM100 electron microscope operating at 60kV. Changes were evaluated in six randomly chosen regions by an investigator blinded for *Drosophila* groups, who <1> calculated the ratio of area positive for myolysis (areas with >10% loss of sarcomeres) compared to total area, using ImagePro software (29); and <2> scored mitochondrial degeneration by determining the ratio degenerated versus total mitochondria.

Calpain activity measurement

Early Drosophila pupae were lysed in a non-denaturing buffer (CelLytic™ MT Cell Lysis Reagent, Sigma, The Netherlands). Protein concentration was determined by using Bio-Rad Protein Assay Kit (Bio-Rad, USA). DABCYL-Thr-Pro–Leu -Lys~Ser-Pro-Pro-Pro-Ser-Pro-Arg-EDANS (Calpain Substrate III, Fluorogenic, Merck, Germany) was used as a substrate for calpain. Hereto, protein extract (30 μg) was added to Calpain Substrate III (60 μM final concentration) in 100 μl phosphate buffered saline. Fluorescence release was measured at 320-nm excitation/460-nm emission by fluorometry (SpectraMax Gemini XPS, Molecular Device, USA) after 90 min incubation at room temperature.

Statistical analysis

Results are expressed as mean± SEM. All experimental procedures were performed in at least duplicate series. ANOVA was used for multiple-group comparisons. Student *t* tests were used for comparisons involving only 2 groups, and *t* tests with Bonferroni correction were used to compare individual group differences when multiple-comparison

ANOVA was significant. All *P* values were two-sided. $P<0.05$ was considered statistically significant. SPSS version 16.0 was used for statistical evaluation.

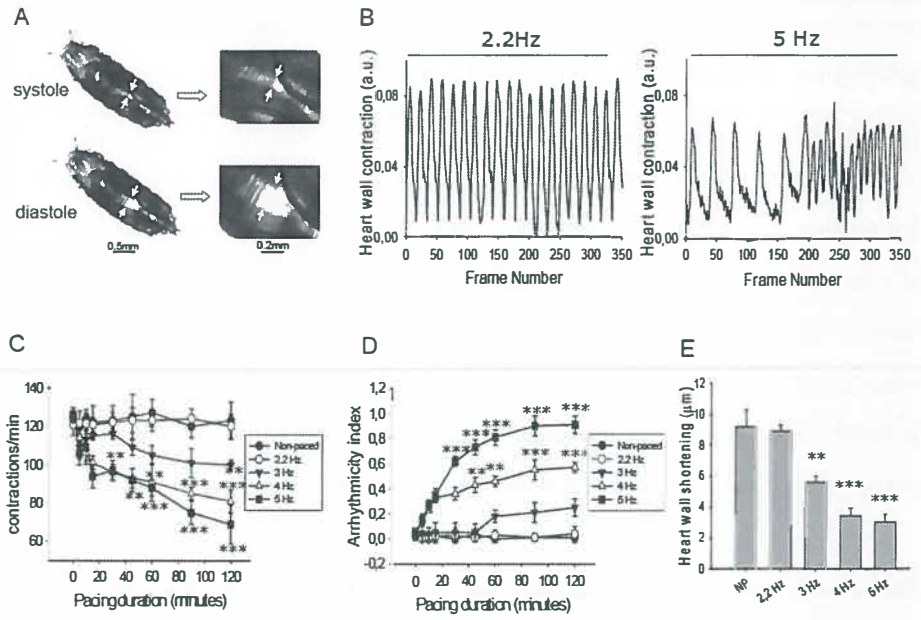


Figure 1 Tachypacing of *Drosophila* induces heart wall remodeling, including gradual reduction in contractile function, induction in arrhythmic periods and reduction in heart wall shortening. **A)** Images of an early Drosophila pupae in systole and diastole. The heart wall is marked with an arrow. **B)** Illustrations of heart wall contractions are depicted after 2 hours normal pacing (2,2Hz) and tachypacing (5Hz) of early pupae of a W1118 genetic background. 350 frames corresponds to 10 seconds. **C)** Rate of heart wall contractions are depicted for non paced, normal paced (2,2Hz) and tachypaced (3,4 and 5Hz) early pupae. **D)** Arrhythmicity index of non-paced, normal paced (2.2Hz) and tachypaced (3,4 and 5Hz) early pupae. **E)** Amplitude of heart wall shortening of normal paced (2,2Hz) or tachypaced (3,4 and 5Hz, 2 hours) wild-type early pupae. N=13-20 early pupae per condition. ** $P<0.01$ vs Control normal paced, *** $P<0.001$ vs Control normal paced.

Results

Tachypacing of *Drosophila* induces contractile dysfunction in the heart wall.

Early *Drosophila* pupae (Figure 1A) possessed a basal rate of 2.2Hz ± 0.2Hz and were subjected to tachypacing at 3, 4 and 5 Hz for up to 2 hours. During interruption of tachypacing, the spontaneous contractile function of the heart wall was determined. It was found that tachypacing results in a gradual and significant reduction in heart contraction rate (Figure 1B,C, supplementary information movie 1 and 2). In addition, tachypacing also induced a significant induction in arrhythmic episodes (Figure 1B,D, supplementary information movie 3 and 4). Moreover, increasing frequency of tachypacing induced a gradual reduction in the amplitude of diastolic and systolic heart wall shortening (Figure 1E). Since tachypacing at 5Hz, a 2.3 fold rate increase compared to basal heart rate, significantly induced contractile dysfunction of the heart wall, this setting was used in all following tachypacing experiments.

Heat shock and the HSP-inducers GGA and BGP-15 increase HSP levels in *Drosophila* and protect against tachypacing-induced contractile dysfunction.

In *in vitro* cardiomyocyte and *in vivo* canine models for AF, it was observed that HSP-inducing treatments protect against tachycardia remodeling (4,8). In order to determine if the same result can be obtained in tachypaced *Drosophila*, fly stocks were subjected to a heat treatment (1 hour 37°C, 16 hours recovery), or pretreatment with the HSP inducing agents GGA or BGP15. Quantitative polymerase chain reaction with reverse transcription (RT–PCR) was used to determine the relative abundance of endogenous DmHSPs. All three HSP-inducing treatments effectively induced the heat shock response as evidenced by an increase in mRNA levels of endogenous DmHSP70AA and DmHSP27 (Figure 2A).

All HSP inducing treatments protected against tachypacing-induced reduction in heart

contraction rate (Figure 2B), increase in arrhythmic episodes (Figure 2C) and reduction in amplitude of heart wall shortening (Figure 2D). These results indicate that boosting the heat shock response results in protection against tachypacing-induced contractile dysfunction of the heart in *Drosophila* pupae, thus further validating *Drosophila* as an *in vivo* model for tachycardia remodeling.

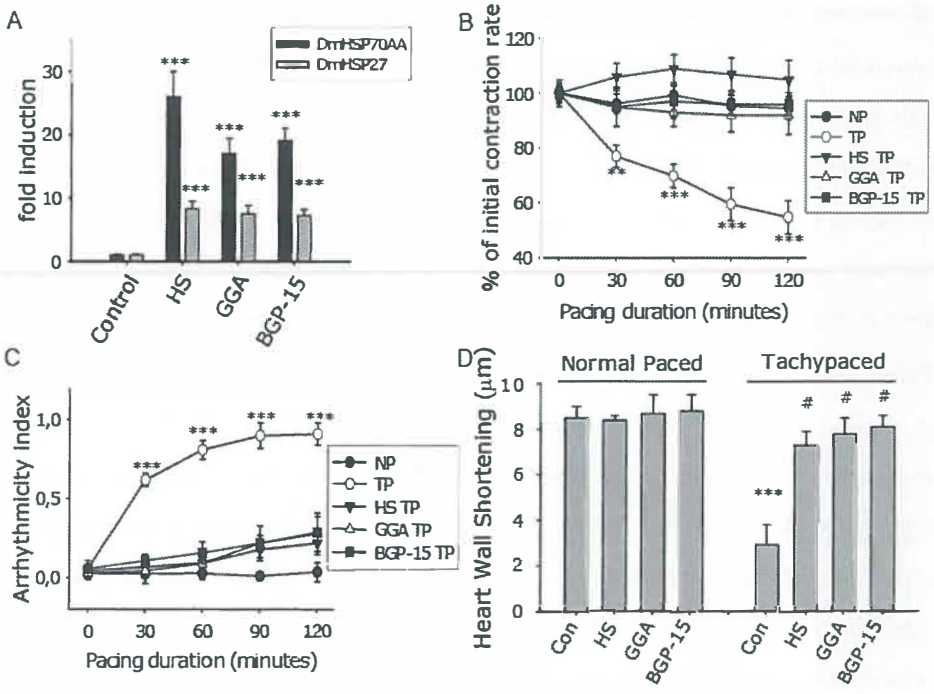


Figure 2 HSP-inducing treatments protect against tachycardia remodeling in *Drosophila*. **A)** A mild heat shock (HS), GGA or BGP-15 pretreatment induces the amount of mRNA of *Drosophila* DmHSP27 and DmHSP70AA compared to RpL32 levels as a standard, as determined by QPCR. **B)** The percentage of initial contraction rate is depicted for normal paced (NP) and tachypaced (TP) early W1118 pupae with or without HS, GGA or BGP-15 pretreatment. **C)** Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae with or without HS, GGA or BGP-15 pretreatment. **D)** Degree of heart wall shortening of normal paced or tachypaced (2 hours) wild-type early pupae with or without HS, GGA or BGP-15 pretreatment. N=15-20 early pupae per condition. ** $P<0.01$ vs Control, ***$P<0.001$ vs Control, # $P<0.01$ vs Control tachypaced.

DmHSP23 overexpression protects against tachypacing-induced contractile dysfunction and structural changes of the heart wall.

Previously, we observed a protective effect of HSPB1, the most prominent heat inducible cytosolic member of the human family of small HSPs, against tachycardia remodeling in HL-1 atrial cardiomyocytes (4). To further validate our model, it was tested if the cytosolic heat inducible member of the *Drosophila* family of small HSPs, DmHSP23 (21,30), protects against tachycardia remodeling. Indeed, the DmHSP23 overexpressing transgenic strain (Table 1) was protected against tachypacing-induced reduction in heart contraction rate (Figure 3A,B, supplementary movie 3), increase in duration of arrhythmic episodes (Figure 3C) and also reductions in amplitude of heart wall shortening (Figure 5C). In addition, (ultra)structural evaluation of the heart wall indicates that tachypacing induces loss of sarcomeres (myolysis, Figure 4A,B) and degeneration of the mitochondria (Figure 4A,C), including occasional enlargement and disorganization of cristae (Figure 4A). All these (ultra)structural changes were significantly attenuated in tachypaced DmHSP23 overexpressing *Drosophila* strain (Figure 4B,C). Previously, it was shown that induced calpain activity plays a molecular switch between the initial trigger for functional remodeling, calcium overload, and structural remodeling in experimental models for AF, but also in human AF (5,31). Therefore, calpain activity was measured in normal paced and tachypaced *Drosophila*. It was observed that tachypacing induces an increase in calpain activity, which was significantly attenuated in the DmHSP23 overexpressing *Drosophila* strain (Figure 4D).

Unravelling the mechanism of DmHSP23 protection against tachycardia remodeling, by comparing small HSP overexpressing Drosophila strains.

Having established that tachypacing of *Drosophila* results in contractile dysfunction, which can be prevented by general overexpression of HSPs and in particular DmHSP23, we next used a genetic approach to get more mechanistic insight in its mode of protection.

Hereto, we used five *Drosophila* strains overexpressing different small HSPs with various cellular localization and functions in protein refolding, protein aggregation and autophagy (Table 1). Overexpression of DmCG4461, which lacks these functions, was also ineffective in protection against tachycardia remodeling (Figure 5). Next, it was tested whether the protective effect of DmHSP23 requires it cytosolic localization. Hereto, we used flies overexpressing DmHSP27, which is exclusively localized in the nucleus but otherwise shares most (functional) features of DmHSP23 (28,30,32-34). As can be seen in Figure 3B,C DmHSP27 did not result in protection against AF, indicating that a protein refolding activity in the nucleus is insufficient for the protective effect. Yet, overexpression of the DmCG7409, a cytosolic protein that has a strong activity to support protein refolding, did also not reveal tachycardia protection, suggesting that the remodeling induced by tachycardia cannot be prevented by the refolding-like activities of DmCG7409. Overexpression of DmHSP67Bc, which lacks this classical chaperone-like activity (Table 1) (25) but which induces autophagy and hereby assists in the clearance of toxic protein aggregates (24), also did not protect against tachycardia remodeling (Figure 5). In addition, overexpression of (the non-heat inducible) DmCG14207, which enhances protein refolding (25) and associates with Z bands in muscles (35), did not protect against tachycardia remodeling (Figure 5). Overexpression of the small HSP of interest was confirmed by Western blot analysis (Figure 6). Thus, our findings suggest that the effectiveness of DmHSP23 to protect against tachycardia remodeling is due to a specific cytosolic feature related to tachycardia, which is unrelated to protein refolding or autophagic capacities, and can not be prevented or repaired by all other small HSP members.

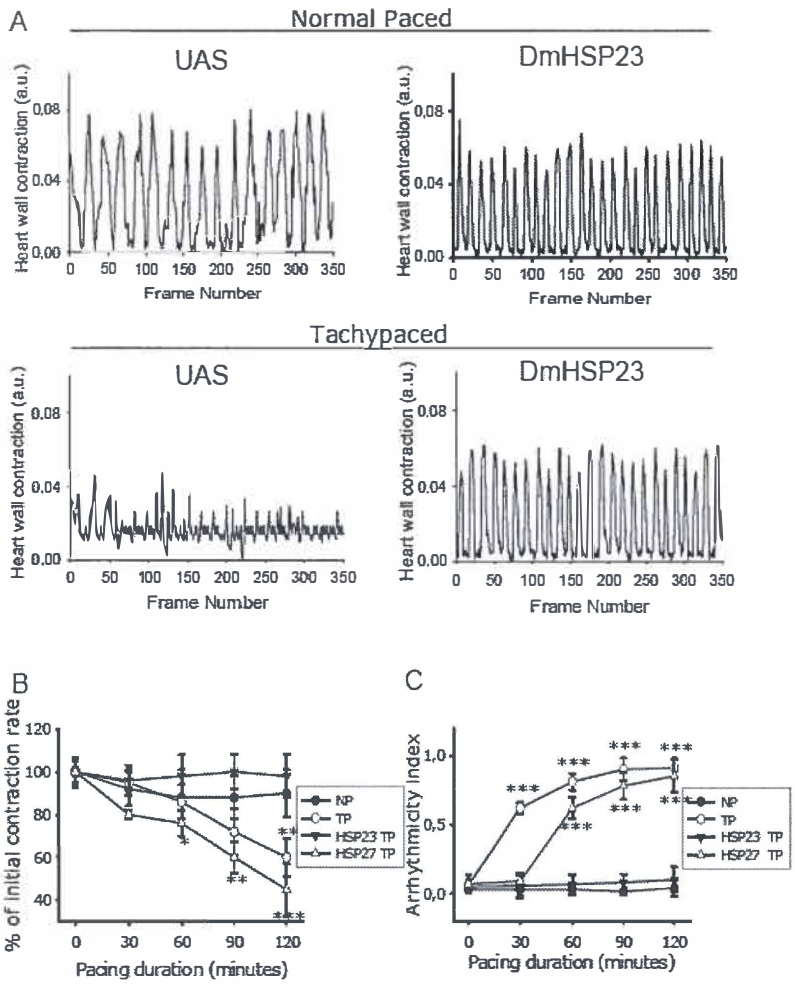


Figure 3. DmHSP23 but not DmHSP27 overexpressing *Drosophila* are protected against tachycardia remodeling. **A)** Heart wall contractions are depicted for normal paced (NP) and tachypaced (TP, 2 hours) early pupae with UAS-DmHSP23 genetic background (no DmHSP23 overexpression, UAS) and with a UAS-actinGAL4-DmHSP23 (DmHSP23) overexpressing background. **B)** The percentage of initial contraction rate is depicted of normal paced (NP) and tachypaced (TP) early pupae of control UAS, DmHSP23 and DmHSP27 overexpressing strains. **C)** Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae of control UAS, DmHSP23 and DmHSP27 overexpressing strains. N=12-20 early pupae per condition. **P*<0.05, ** *P*<0.01 vs Control, ****P*<0.001 vs Control.

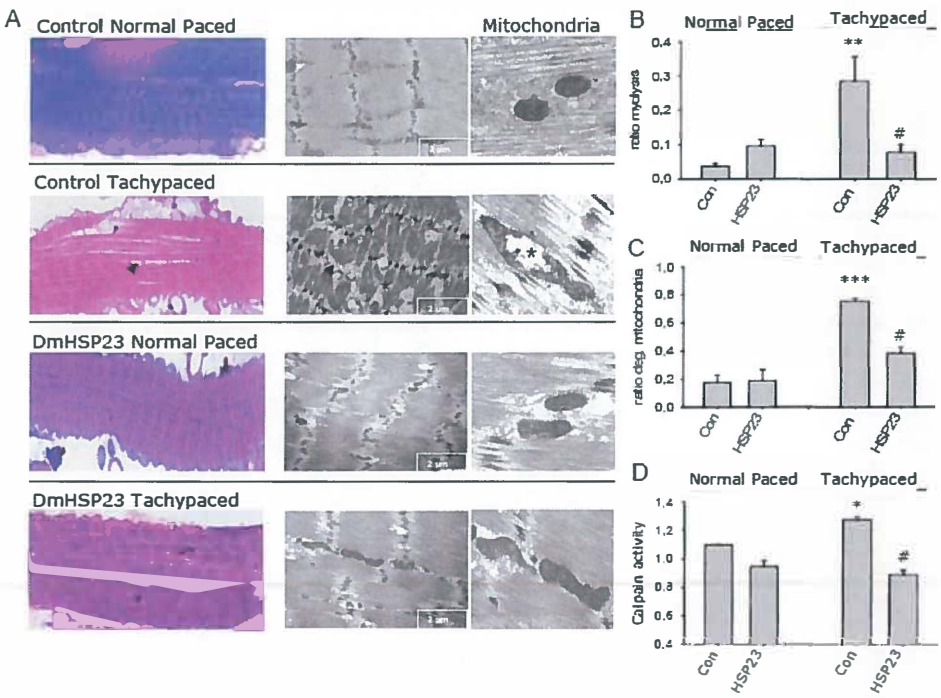


Figure 4 DmHSP23 overexpressing *Drosophilas* are protected against tachypacing-induced structural remodeling, including loss of sarcomeres (myolysis) and mitochondrial damage. **A)** Left panel shows light microscopic structure of the heart wall in normal paced and tachypaced control (UAS-DmHSP23) and DmHSP23 (UAS-actinGAL4-DmHSP23) overexpressing strains. Right panel shows electron microscopic details of the cardiomyocytes and mitochondria. Arrowheads show loss of sarcomeres in the heart wall and asterisk marks mitochondrial damage. **B)** Quantification of ratio myolysis compared to total area of sarcomeres and **C)** ratio degenerated mitochondria compared to total amount of mitochondria in normal paced and tachypaced control (UAS-DmHSP23) and DmHSP23 (UAS-actinGAL4-DmHSP23) overexpressing strains. **D)** Tachypacing induces calpain activity, and DmHSP23 (UAS - actinGAL4 - DmHSP23) overexpressing strains were protected. N=3-5 early pupae per condition $^{**}P<0.01$ vs Control normal paced , # $P<0.05$ vs Control tachypaced.

Figure 5 HSP67Bc, CG4461, CG7409 and CG14207 show no protective effects against tachycardia remodeling in *Drosophila*. **A)** The percentage of initial contraction rate is depicted for normal paced (NP) and tachypaced (TP) early pupae of control W1118-actinGAL4 and UAS-actinGal4-HSP67Bc, -CG4461, -CG7409 or -CG14207 overexpressing strains. **B)** Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae of control and HSP67Bc, CG4461, CG7409 or CG14207 overexpressing strains. **C)** Combined results of degree of heart wall shortening of normal paced or tachypaced (2 hours) early pupae of control and DmHSP23, DmHSP27, HSP67Bc, CG4461, CG7409

or CG14207 overexpressing strains. N=11-15 early pupae per condition. ***P<0.001 vs Control NP, # P<0.001 vs Control TP.

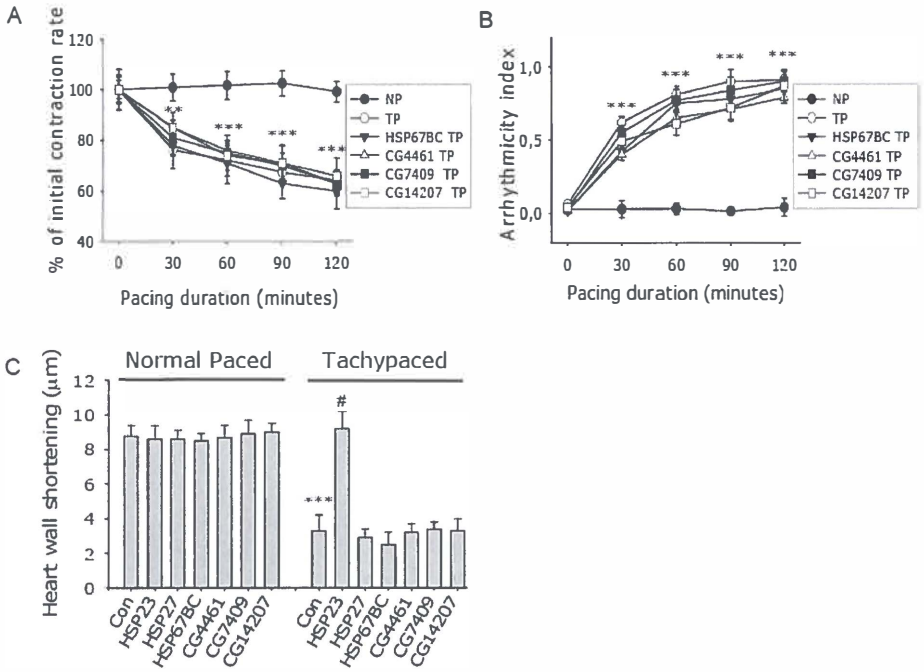


Table 1 Overview of Drosophila melanogaster transgenic small HSP overexpressing lines used in the current study.

Drosophila small HSP	Name	Function	Reference
Classical sHSP	HSP23	-heat inducible	(25,30)
		-assists protein refolding	
	HSP27	-heat inducible	(25,28,30)
		-assists protein refolding	
Novel sHSPs	HSP67Bc	-heat inducible	(24,25)
		-prevents protein aggregation	
		-induces autophagy	
	CG4461	-heat inducible	(25)
	CG7409	-mild heat inducible	(25)
		-assists protein refolding	
	CG14207	-not heat inducible	(25)
		-assists protein refolding	

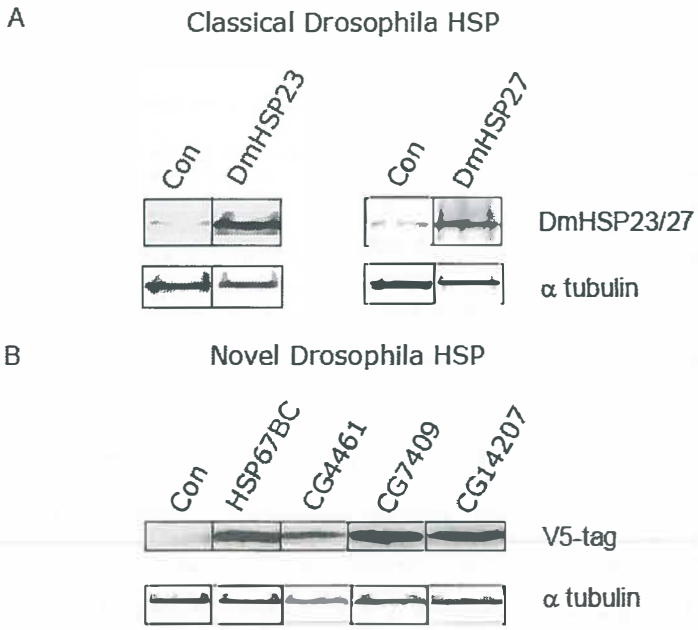


Figure 6 Western blot showing overexpression of the individual small DmHSPs in transgenic *Drosophila* strains. **A)** Representative blot showing overexpression of the classical *Drosophila* small HSPs, DmHSP23 (UAS-actinGAL4-DmHSP23) and DmHSP27 (UAS-actinGAL4-DmHSP27), compared to respectively control UAS-DmHSP23 or UAS-DmHSP27 genetic background. **B)** Representative blot showing overexpression of novel *Drosophila* small HSPs, UAS-actinGAL4-HSP67Bc, -CG4461, -CG7409 or -CG14207, compared to control actinGAL4-W1118 genetic background. Alpha-tubulin was used as a loading control.

Discussion

Previously, we reported on cardioprotective effects of HSPs, especially the small human HSPB1, and the HSP-inducing agent GGA against important features of tachycardia remodeling, such as electrical and structural remodeling and contractile dysfunction (4,6-8). In the present study, we demonstrated for the first time that the protective effects are also observed *in vivo* in the *Drosophila melanogaster* model for tachypacing-induced contractile dysfunction of the heart wall. We observed that both HSP-inducing agents GGA and BGP-15 but also a heat shock pretreatment protect against tachypacing-induced contractile dysfunction. By comparing in transgenic *Drosophila* strains the overexpression of six individual small HSPs, it was found that only DmHSP23 resulted in cardioprotective effects. Moreover, our findings indicate that similar findings observed in *in vitro* tachypaced HL-1 and dog atrial cardiomyocytes and *in vivo* canine model for AF, *Drosophila melanogaster* can be used to study tachycardia remodeling. Since *Drosophila* can be easily utilized to manipulate gene expression in a highly precise spatial and temporal fashion, this model of AF thus seems to represent an excellent tool for studying molecular mechanisms of heat shock-mediated cardioprotection and genes involved in tachycardia remodeling and AF progression.

Tachypaced *Drosophila melanogaster* as model system for contractile dysfunction of the heart wall.

The *Drosophila* heart is a simple tube that runs along the dorsal midline of the pupae (36). The heart is composed of two major cell types: cardioblasts, which form the heart tube and are the contractile cells of the heart, and pericardial cells, which flank the cardioblasts but do not express muscle proteins (36). Moreover, *Drosophila* myocardium contains myogenic cardiac pacemaker cells, which respond to a variety of neurotransmitters and hormones by adjusting heart rate (37). Tachypacing of the *Drosophila* heart induced contractile dysfunction of the heart wall that was comparable to

the remodeling observed in *in vitro* tachypaced cardiomyocytes (5,38), *in vivo* animal models for AF (39,40) and clinical AF (41,42). Moreover, the cardioprotective effect of the general induction of the heat shock response, that was previously shown to be protective in *in vitro* (4,6) and *in vivo* (4,8) AF models, was also found to be protective in the tachypaced *Drosophila* model. Finally, also overexpression of a single small HSP, DmHSP23, revealed the same cardioprotective effects as seen for human HSPB1 in tachypaced HL-1 cardiomyocytes. Together, the data validate the tachypaced *Drosophila* model as versatile model system for tachycardia-induced heart wall remodeling and AF progression.

Small heat shock proteins in *Drosophila melanogaster*

The family of *Drosophila melanogaster* small HSPs comprises 12 proteins, but only four of them have been studied in greater detail and form the classical DmHSPs, DmHSP22, DmHSP23, DmHSP26 and DmHSP27 (25). Although these four proteins share high homology, their intracellular localization differs (25,30). DmHSP22 is localized to mitochondria (43), DmHSP23 and DmHSP26 were found in the cytosol (30,32) and DmHSP27 is present in the nucleus (33). It is unknown which member of the *Drosophila* small HSP family represents the functional ortholog of human HSPB1. Based on the heat inducibility of HSPB1, its cytoplasmic localization, and its ability to function in the HSPA1A dependent refolding activity, DmHSP23 and DmHSP26 most closely resemble HSPB1 (25). DmHSP26 is, however, rather effective in protection against aggregation of polyglutamine proteins that are associated with neurodegenerative disease (30), a property that is lacking for DmHSP23 (30) and human HSPB1 (44). Although we did not include flies overexpressing DmHSP26, we now show that DmHSP23, like HSPB1, also protects against tachycardia remodeling. This finding suggests that DmHSP23 might represent a functional ortholog of human HSPB1.

The question arised which small HSP activity is required for cardioprotection in AF. In a

recently completed study comparing all 10 human HSPB members in a HL-1 cardiomyocyte model for AF (45), we found three members, HSPB1, HSPB6, and HSPB7, to strongly protect against tachycardia remodeling and one member, HSPB8, had minor protective actions. DmHSP67Bc was recently found to be the functional ortholog of the human HSPB8 (24), and was ineffective in the current study. This implies that the autophagy stimulating function of the human HSPB8 (24,46,47) and DmHSP67Bc (24) is insufficient to protect against tachycardia remodeling in *Drosophila*. The function shared by HSPB1, HSPB6, and HSPB7 that was found responsible for cardioprotection, was their ability to bind to actin and to prevent actin polymerization (45) and not their association with canonical activities of HSPBs like refolding of denatured proteins (45). Also in the current study, two cytosolic members of the *Drosophila* small HSP family that do function in refolding, CG7409 and CG14207, do not protect against tachycardia remodeling. This suggests that neither CG7409 nor CG14207 are likely to be functional orthologs of HSPB1, HSPB6, or HSPB7. The same is true for CG4461, for which no clear activity has been found so far. Finally, flies overexpressing DmHSP27, which is exclusively localized in the nucleus but otherwise shares most (functional) features of DmHSP23 (30) did not result in protection against tachycardia remodeling, indicating that cytoplasmic activity is required for a protective effect. Based on the studies with the human HSPB members (45), actin binding may be a key feature required for such cardioprotective activity. Whether DmHSP23 indeed can bind actin has not been demonstrated so far. Only for HSP67Bc and CG14207, it was shown that they associate with Z bands in muscles (24,35)[22;32], but neither one of these two members protected against tachycardia remodeling in *Drosophila*, indicating that this feature is not associated with the observed cardioprotective effect. Furthermore, it might be argued that the V5 tag of the novel small HSPs is suppressing its function, but the HSP67Bc, CG14207 and CG7409 transgenic strains were found to be efficient in preventing aggregation of disease related misfolded proteins, indicating no functional interference of

the V5 tag (25). Thus, the precise mechanism for the effectiveness of DmHSP23 to protect against tachycardia remodeling remains to be determined, but there are indications that a specific tachycardia-related feature of DmHSP23 that is not shared by the other five small DmHSP members, plays a key role. The combined data also demonstrate that, next to sharing common functions in proteostasis, small HSP seem to have distinct client specific functions.

In summary, the tachypaced *Drosophila* model for AF as shown in the current study, provides a versatile system to study both mechanism of tachycardia remodeling and disease progression, to search for genetic modifiers of tachycardia remodeling, and to screen for pharmacological compounds that can prevent AF mediated cardiac damage.

Supplementary materials related to this article can be found online at http://www.sciencedirect.com/science/article/pii/S0022282811002288

Acknowledgements

Authors like to thank Martha Ritsema, Bart Kanon, Michel Vos and Joris Parmentier for excellent technical assistance and Dr. Herman Steen (CEO Nyken BV) for critically reviewing the manuscript.

This study was supported by the Dutch Organization for Scientific Research (NWO program grant 916. 46. 043), Ubbo Emmius Bursaal grant (UMCG Number 800403), ERFRO grant (Operationeel Programma Noord-Nederland 2007-2013 (OP-EFRO), Drug delivery en targeting cluster) and Dutch Heart Foundation (2009B024) and a grant from the Canadian Institutes of Health Research to RMT (MOP- 77796).

References

1. Nattel S. New ideas about atrial fibrillation 50 years on. *Nature* **2002**;415:219-226.

2. Schoonderwoerd BA, Van Gelder IC, Van Veldhuisen DJ, Van Den Berg M, Crijns HJGM. Electrical and structural remodeling: role in the genesis and maintenance of atrial fibrillation. *Porg Cardiovasc Dis* **2005**;48:153-168.

3. Casaclang-Verzosa G, Gersh BJ, Tsang TS. Structural and functional remodeling of the left atrium: clinical and therapeutic implications for atrial fibrillation. *J Am Coll Cardiol* **2008**;51:1-11.

4. Brundel BJJM, Shiroshita-Takeshita A, Qi XY, *et al*. Induction of heat-shock response protects the heart against atrial fibrillation. *Circ Res* **2006**;99:1394-1402.

5. Brundel BJJM, Kampinga HH, Henning RH. Calpain inhibition prevents pacing induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. *Cardiovasc Res* **2004**;62:521-528.

6. Brundel BJJM, Henning RH, Ke L, Van Gelder IC, Crijns HJGM, Kampinga HH. Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation. *J Mol Cell Cardiol* **2006**;41:555-562.

7. Brundel BJJM, Ke L, Dijkhuis AJ, *et al*. Heat shock proteins as molecular targets for intervention in atrial fibrillation. *Cardiovasc Res* **2008**;78:422-428.

8. Sakabe M, Shiroshita-Takeshita A, Maguy A, *et al*. Effects of heat shock protein induction on atrial fibrillation caused by acute atrial ischemia. *Cardiovasc Res* **2008**;78:63-70.

9. Grobety M, Sedmera D, Kappenberger L. The chick embryo heart as an experimental setup for the assessment of myocardial remodeling induced by pacing. *Pacing Clin Electrophysiol* **1999**;22:776-782.

10. Yue L, Feng J, Gaspo R, Li GR, Wang Z, Nattel S. Ionic remodeling underlying action potential changes in a caninemodel of atrial fibrillation. *Circ Res* **1997**;81(4):512-525.

11. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation* **1995**;92(7):1954-1968.

12. Leistad E, Aksnes G, Verburg E, Christensen G. Atrial contractile dysfunction after short-term atrial fibrillation is reduced by verapamil but increased by BAY K8644. *Circulation* **1996**;93(9):1747-1754.

13. Bier E, Bodmer R. Drosophila, an emerging model for cardiac disease. *Gene* **2004**;342:1-11.

14. Wolf MJ, Amrein H, Izatt JA, Choma MA, Reedy MC, Rockman HA. Drosophila as a model for the

identification of genes causing adult human heart disease. *Proc Natl Acad Sci USA* **2006**;103:1394-1399.

15. Wessells RJ, Fitzgerald E, Cypser JR, Tatar M, Bodmer R. Insulin regulation of heart function in aging fruit flies. *Nat Genet* **2004**;36:1275-1281.

16. Yi P, Han Z, Li X, Olson E. The mevalonate pathway controls heart formation in Drosphila by isoprenylation of G γ 1. *Science* **2006**;313:1301-1303.

17. Neely GG, Kuba K, Cammarato A, *et al.* A global in vivo Drosophila RNAi screen identifies NOT3 as a conserved regulator of heart function. *Cell* **2010**;141:142-153.

18. Ocorr KA, Crawley T, Gibson G, Bodmer R. Genetic variation for cardiac dysfunction in Drosophila. *PlosOne* **2007**;2:e601.

19. Cammarato A, Dambacher CM, Knowles AF, *et al.* Myosin transducer mutations differentially affect motor function, myofibril structure, and the performance of skeletal and cardiac muscles. *Mol Biol Cell* **2008**;19:553-562.

20. Kim IM, Wolf MJ. Serial examination of an inducible and reversible dilated cardiomyopathy in individual adult Drosophila. *PlosOne* **2009**;4:e7132.

21. Michaud S, Marin R, Tanguay RM. Regulation of heat shock gene induction and expression during Drosophila development. *Cell Mol Life Sci* **1997**;53:104-113.

22. Tanguay RM, Morrow G. Neural expression of small heat shock proteins influences longevity and resistance to oxidative stress. Heat shock proteins and the brain: implications for neurodegenerative diseases and neuroprotection. *Springer Science+Business Media*; **2008**. p. 319-336.

23. Wang HD, Kazemi-Esfarjani Benzer S. Multiple-stress analysis for isolation of Drosophila longevity genes. *Proc Natl Acad Sci* **2004**;101:12610-12615.

24. Carra S, Boncoraglio A, Kanon B, *et al.* Identification of the Drosophila ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J Biol Chem* **2010**;285:37811-37822.

25. Vos MJ. Small heat shock proteins; implications for neurodegeneration and longevity. *Thesis*. **2009**.

26. Wessells RJ, Bodmer R. Screening assays for heart function mutants in Drosophila. *Biotechniques* **2004**;37:58-66.

27. Marin R, Valet JP, Tanguay RM. Hsp23 and Hsp26 exhibit distinct spatial and temporal patterns of constitutive expression in Drosophila adults. *Dev Genet* **1993**;14:69-77.

28. Marin R, Tanguay RM. Stage-specific localization of the small heat shock protein Hsp27 during oogenesis in Drosophila melanogaster. *Chromosoma* **1996**;105:142-149.

29. Brundel BJJM, Ausma J, Van Gelder IC, *et al*. Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res* **2002**;54:380-389.

30. Morrow G, Heikkila JJ, Tanguay RM. Differences in the chaperone-like activities of the four main small heat shock proteins of Drosophila melanogaster. *Cell Stress Chaperones* **2006**;11:51-60.

31. Ke L, Qi XY, Dijkhuis AJ, *et al*. Calpain mediates cardiac troponin degradation and contractile dysfunction in atrial fibrillation. *J Mol Cell Cardiol* **2008**;45:685-693.

32. Beaulieu JF, Arrigo AP, Tanguay RM. Interaction of Drosophila 27,000 Mr heat shock protein with the nucleus of heat-shocked and ecdysone-stimulated culture cells. *J Cell Sci* **1989**;92:29-36.

33. Michaud S, Lavoie S, Guimond MO, Tanguay RM. The nuclear localization of Drosophila Hsp27 is dependent on a monopartite arginine-rich NLS and is uncoupled from its association to nuclear speckles. *Biochem Biophys Acta* **2008**;1783:1200-1210.

34. Marin R, Landry J, Tanguay RM. Tissue-specific posttranslational modification of the small heat shock protein HSP27 in Drosophila. *Exp Cell Res* **1996**;223:1-8.

35. Arndt V, Dick N, Tawo R, *et al*. Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr Biol* **2010**;20:143-148.

36. Ward EJ, Skeath JB. Characterization of a novel subset of cardiac cells and their progenitors in the Drosophila embryo. *Development* **2000**;127:4959-4969.

37. Johnson E, Sherry T, Ringo J, Dowse H. Modulation of the cardiac pacemaker of Drosophila: cellular mechanisms. *J Comp Physiol* **2002**;172:227-236.

38. Sun H, Chartier D, Leblanc N, Nattel S. Intracellular calcium changes and tachycardia-induced contractile dysfunction in canine atrial myocytes. *Cardiovasc Res* **2001**;49:751-761.

39. Wakili R, Yeh YH, Yan Qi X, *et al*. Multiple potential molecular contributors to atrial hypocontractility caused by atrial tachycardia remodeling in dogs. *Circ Arrhythm Electrophysiol* **2010**;3:530-541.

40. Lenaerts I, Heinzel FR, Driesen RB, *et al*. Ultrastructural and functional remodeling of the coupling between Ca2+ influx and sarcoplasmic reticulum Ca2+ release in right atrial myocytes from experimental persistent atrial fibrillation. *Circ Res* **2009**;105:876-885.

41. Manning WJ, Silverman DI, Katz SE, *et al.* Impaired left atrial mechanical function after cardioversion: relation to the duration of atrial fibrillation. *J Am Coll Cardiol* **1994**;23(7):1535 - 40.

42. Daoud EG, Marcovitz P, Knight B, *et al.* Short-term effect of atrial fibrillation on atrial contractile function in humans. *Circulation* **1999**;99:3024-3027.

43. Morrow G. The small heat shock protein Hsp22 of Drosophila melanogaster is a mitochondrial protein displaying oligomeric organization. *J Biol Chem* **2000**;275(40):31204-31210.

44. Vos MJ, Zijlstra MP, Carra S, Sibon OC, Kampinga HH. Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy* **2011**;7:101 - 3.

45. Ke L, Meijering RAM, Hoogstra-Berends F, *et al.* HSPB1, HSPB6, HSPB7 and HSPB8 protect against RhoA GTPase-induced remodeling in tachypaced HL-1 atrial myocytes. *PLos One* **2011**;6:e20395.

46. Carra S, Seguin SJ, Lambert H, Landry J. HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem* **2008**;283:1437-1444.

47. Carra S, Brunsting JF, Lambert H, Landry J, Kampinga HH. HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2{alpha} phosphorylation. *J Biol Chem* **2009**;284:5523-5532.

CHAPTER 6

General Discussion and Future Perspectives

1. Central concepts of atrial remodeling

Atrial fibrillation (AF) is the most common sustained clinical tachyarrhythmia and a significant contributor to cardiovascular morbidity and mortality (1). AF has the tendency to become more persistent over time (2 - 4). In addition, the longer the arrhythmia exists, successful pharmacological and electrical cardioversion, including the maintenance of sinus rhythm thereafter, is more difficult (3). Clinical observations show that immediately after cardioversion, atrial contractile function is severely impaired or even absent and recovery of contractile capacity is related to the preceding AF duration (1).

Over the past decade, much research has focused on the dissection of mechanisms underlying the progressive nature of AF (1). An important recognition was that AF, once initiated, alters atrial electrophysiological properties in a manner that favors the induction and maintenance of AF (2). A conceptual model for the mechanisms underlying AF progression is depicted in Figure 1. During AF, atrial cardiomyocytes are subjected to very rapid (400 - 600 times per min) and irregular activation, causing an excess Ca^{2+} entry via the L-type Ca^{2+} channel into the myocytes (5). The resulting increase in intracellular Ca^{2+} causes inactivation of the L-type Ca^{2+} channel. As a result, the action potential shortens and contractile dysfunction develops, enhancing the likelihood of induction and maintenance of AF (6).

A second mechanism of early remodeling consists of changes in the activity of specific kinases and phosphatases, resulting in the modulation of key intracellular proteins involved in Ca^{2+} handling. **Chapter 4** revealed a prime role for tachypacing-induced RhoA GTPase activity and subsequent F-actin stress fiber formation and reduction in Ca^{2+} transient (CaT). Rho GTPases represent a family of small GTP-binding proteins involved in cytoskeletal organization, migration, transcription, and proliferation and have gained considerable recognition as powerful regulators of actin cytoskeletal organization in the heart (7). It has been recognized that active Rho GTPases stimulate the conversion of G-actin to F-actin, which results in cytoskeletal injury including changes in calcium signaling, disturbances in action potential conduction and contractile dysfunction, which

are all substrates for the development of AF. Actin stress fiber assembly and contraction are predominantly mediated by Rho-associated serine/threonine kinase (ROCK), a major down-stream effector of the Rho pathway. Consistent with all of the above, we show that tachypacing activates RhoA and that inhibition of ROCK, its effector of actin polymerization, prevents tachypacing-induced reduction in CaT (**Chapter 4**).

Meanwhile, by using *in vitro* models, it was also found that the tachypacing-induced decrease in L-type Ca^{2+} current can be mediated by activation of other key phosphorylation-regulating systems, potentially including CaMKII and calcineurin related pathways (8). Furthermore, indirect evidence suggests that AF-induced changes in the kinomic profile leads to de-phosphorylation of the L-type Ca^{2+} channel, and consequently in reduction of the L-type Ca^{2+} current (9,10). In addition, AF is associated with phosphorylation of phospholamban and ryanodine receptors (RyR2), which might endorse cellular Ca^{2+} overload (11,12). So it can be concluded that AF induces changes in the phosphorylation status of various proteins that are involved in atrial remodeling.

When AF persists, structural remodeling of cardiac tissue will occur (13,14). This form of tissue adaptation resembles ischemic hibernation and is defined by the ability of the myocytes to turn into a non-functional phenotype by degradation of the myofibril structure (myolysis), which leads to contractile dysfunction (14 - 17). Cell viability will, however, be maintained for a prolonged period of time, thus ensuring tissue integrity (but not tissue functionality) (16). Consequently, myolysis is found in patients with persistent AF and not in patients who exclusively display paroxysmal forms of the arrhythmia (13), suggesting a role for myolysis in the promotion of remodeling. Previously, it was shown that the cysteine protease calpain represents an important switch between tachycardia-induced Ca^{2+} overload and atrial remodeling (13). **Chapter** 2 describes the first study to provide definite evidence for a main role of calpain in troponin degradation and dysfunction in both *in vitro* tachypaced HL-1 atrial cardiomyocytes and human tissue of patients with AF. Tachypacing of HL-1 cardiomyocytes induced a gradual and significant degradation of various cardiac troponins (cTnI, cTnT and cTnC) which was associated with contractile dysfunction. Both were prevented by inhibition of calpain but

not by inhibition of caspases or the proteasome. In cardiac tissue of patients with persistent AF, a significant degradation in cTnI, cTnT and cTnC was found, which was absent in cardiac tissue from patients with normal sinus rhythm or paroxysmal AF. Degradation of troponins in turn correlated significantly with both calpain activity and the amount of myolysis in the tissues. These results indicate that calpain activation represents a key factor underlying myofibrillar protein degradation, myolysis and contractile dysfunction. Since myolysis and contractile dysfunction contribute to the AF substrate, tachypacing-induced calpain activation may relate to the self-perpetuating nature of AF. In addition, the activation of cysteine proteases is widely known to initiate and execute apoptosis (18). However, particularly in cardiac myocytes, apoptosis is generally not completed. i.e., no DNA fragmentation occurs and cells remain intact. Depending on the intensity of the stress, activation of cysteine proteases may result in atrial remodeling, by degradation of the L-type Ca^{2+} channel (19,20) or by cleavage of myofilament proteins such as cTnI, cTnT, cTnC (21-23), leading to shortening of action potential duration, myolysis and contractile dysfunction and hence persistency of AF (Figure 1). Interestingly, it was observed in **Chapter 2** that caspases are activated as result of tachypacing. Since effective pan-caspase inhibition did not attenuate cardiac troponin degradation and contractile dysfunction, their role in contractile-protein degradation seems of less importance. The results further indirectly support the idea that the molecular, cellular and functional events that contribute to AF progression do not include apoptosis.

2. *Drosophila melanogaster* as a novel experimental model system for studying tachycardia-induced remodeling

A versatile model system is crucial to study the mechanisms underlying AF-related atrial remodeling. Over the past decades, several animal models such as dog, goat, rabbit and sheep have been extensively used to explore underlying mechanisms and potential treatment of AF (24). Due to the low speed and high cost, and difficulty in manipulating the genetics of these organisms, these *in vivo* models are not convenient for experimental manipulations and high-throughput screening. Recently several *in vitro* cell models,

including the tachypaced HL-1 atrial myocytes, have been applied to provide insights into the mechanisms underlying AF progression and development (**Chapter 2 and 3**, 25). In **Chapter 5,** we introduce a novel versatile *in vivo* model for tachycardia remodeling utilizing *Drosophila melanogaster*. *Drosophila* is one of the most popular invertebrate model organisms and has been used extensively in many areas of biological research, especially genetics and development (26). The application of this model is supported by the existence of functionally conserved features between *Drosophila* and humans, including cardiac aging and development of heart failure (27, 28). Combined with short life-cycle, cost efficiency, the powerful techniques for genetic and molecular manipulations, the *Drosophila* system is highly suitable for compound testing (29). In addition, *Drosophila* contains a pumping heart driven by a primary pacemaker located caudally in the heart from which the heart beat propagates interiorly as a wave of peristalsis (30). Based on these considerations, we started to subject flies at early pupae stages to tachypacing and found that their heart function significantly declines. Furthermore, an increase in calpain activity was found which related to structural remodeling, in the form of myolysis and mitochondrial damage. All these observations are in line with human and experimental AF (14,25). Finally, our tachypacing-induced *Drosophila* system was found to be applicable for chemical compound screening by using HSP-inducing drugs, such as GGA and BGP-15 (**Chapter 5**).

3. HSPB family: intrinsic protection against tachypacing-induced atrial remodeling

Since it has been generally recognized that heat shock proteins (HSPs) reveal protective roles against various forms of stress-induced cell damage (31), we investigated in **Chapter 3** the role of various HSPs in AF by determining the expression levels of a number of key stress-inducible HSPs in atrial tissue of patients with paroxysmal and/or persistent AF. We found elevated HSPB1 expression in atrial tissue from patients with paroxysmal AF compared with patients with persistent AF or sinus rhythm. This was confirmed in a subsequent study by Yang *et al.* (32), suggesting that the ability to up-regulate HSPB1 may be crucial in attenuating the progressive nature of AF. Consistently, an inverse correlation was observed between HSPB1 expression and the

duration of AF and the extent of myolysis (**Chapter 3**). It was therefore hypothesized that increased HSPB1 expression levels limit the progression from paroxysmal to persistent AF.

To test more directly whether HSPs indeed protect against AF promoting effects, the tachypaced HL-1 cell model for AF (**Chapter 3**) and our novel tachypaced *Drosophila melanogaster* model system were used (**Chapter 5**) (25,33). In the HL-1 atrial cardiomyocytes our group previously already observed that pre-induction of HSPs by a mild non-toxic heat shock or by the drug geranylgeranylacetone (GGA) prior to tachypacing prevented atrial remodeling, such as contractile dysfunction and structural remodeling (**Chapter 3**, 25). Consistently, orally administered GGA suppressed AF-related refractoriness abbreviation and AF promotion in dogs (25) and also protected from AF-related contractile dysfunction in flies (**Chapter 5**).

Using the cell model (25, **Chapter 3**), we already demonstrated that this GGA-mediated protection required HSPB1, but not HSPA1A, overexpression. Furthermore, these studies revealed that the single overexpression of HSPB1 sufficed to protect AF-mediated myolysis. This is why we next tested which members of the small HSPB family show protective effects against AF progression and their mode of action.

4. Mode of action of HSPB1 and other HSPB members in AF

In **Chapter 4,** we describe that in addition to HSPB1, also other members of the HSPB family (i.e. HSPB6, HSPB7 and HSPB8) display protective effects against tachypacing-induced remodeling. As several HSPB proteins can form hetero-oligomeric complexes with each other, we first tested whether their protective effect were merely in the context of supporting oligomeric structures of endogenous HSPB1 or not. As downregulation of endogenous HSPB1 did not impair the protective effects of HSPB6, HSPB7 and HSPB8, we concluded that their effects were independent of endogenous HSPB1. Interestingly, all the 4 protective HSPB members (but not the non-protective members) were able to reduce the formation of F-actin stress fibers, supporting the view

that actin protection is the key to the protective effects of HSPB members in AF. Yet, the mode of action in prevention of F-actin formation of the 4 different protective HSPB members seemed to differ. Whereas HSPB8 interfered with the upstream tachypacing-induced RhoA GTPase activitation, HSPB1, HSPB6, and HSPB7 did not. Rather, HSPB1, HSPB6 and HSPB7 were found to directly inhibit G- to F-actin polymerization and/or stimulate depolymerization, indicating a protective role against tachycardia remodeling downstream of RhoA GTPase activation (Figure 1).

Although our data points to actin as a target for HSPB-mediated protection in AF, we do not wish to exclude alternative modes of actions, that even could act in parallel. Such additional mechanisms of protection from AF might involve the direct modulation of ion-channel function or modulation of specific kinases, resulting in the preservation of the L-type Ca^{2+} current. Previously, HSPs were implicated in the regulation of ion-channel function in heart and brain (39-42). Some HSPs were found to interact directly with ion-channels, such as HSPB5 with Na^{+} channels (42), HSPA1A with cardiac K^{+} channel hHERG (40), and voltage-gated Ca^{2+} channels (41). Yet, HSPB5 was not protective in our AF cell model, which makes this mode of action less likely.

HSPBs also may affect several signaling cascades that are activated in AF. E.g., it has been demonstrated in brain epithelial cells that exogenous administration of HSPB1 inhibited the ATP-sensitive and calcium-activated potassium channels (39). Interestingly, studies showed that HSPs, including phosphorylated HSPB1, link signal-transduction cascades to (ion-channel) function (41,43,44) and this might also apply for the protective effect observed against tachypacing-induced reduction in L-type Ca^{2+} current. Although we describe in **Chapter 4** the modulatory action of HSPB8 on RhoA-GTP activity with subsequent inhibition of ROCK and F-actin formation, in general, the exact role of HSPBs in modulating the activation of kinases or phosphatases is unknown. HSPB1 associates with certain (downstream) kinases, such as IkB kinase and c-Jun N-terminal kinase (JNK), thereby suppressing activation of the transcription factor NF-kB (45,46). Interestingly, these kinases were reported to be modulated during AF (47,48). Finally, the protective HSPBs might prevent myocyte remodeling via inhibition of calpain. Although

no studies described the modulation of calpain by HSPB, HSPB1 was found to modulate *in vitro* other cystein proteases such as caspase 3 (49).

Thus, based on the current knowledge, the main target of preventing AF-induced atrial remodeling is via attenuation of F-actin fiber formation. Alternative mechanisms might include prevention of the activation of the cysteine protease calpain, and prevention of changes in kinomics.

Having established that at least 4 HSPB members could protect against tachypacing-induced remodeling, we next tried to test whether this finding could also be translated to the *in vivo* situation. Hereto, we used the *Drosophila melanogster* model for AF that we developed (**Chapter 5**). In order to avoid cross-species related artifacts, we focused on the family of *Drosophila melanogaster* small HSP members. This family comprises of 12 proteins and for several of them transgenic fly lines were already available (DmHSP22, DmHSP23, DmHSP26 and DmHSP27 (50) and CG14207, Hsp67Bc (51). Our findings in **Chapter 5** reveal that only DmHSP23 can protect against AF. This member likely represents a functional ortholog of the human HSPB1 as it shares many of the features of HSPB1 including chaperone activity (35,52,53), dynamic oligomerization in phosphorylation dependent manner (53), cytoskeletal protection (53, This thesis) and now also protection against AF (**Chapter 5**). Another member that we tested, DmHsp67Bc, was recently found to be a functional ortholog of the human HSPB8 (54). Our finding that Hsp67Bc, unlike *in vitro*, did not protect against tachycardia remodeling in *Drosophila* (**Chapter 5**) implies that the RhoA-GTP effects of HSPB8 observed *in vitro* may be insufficient to yield full protection *in vivo*. In this respect, it is noteworthy that also *in vitro* HSPB8 was the weakest of all protective HSPB members *in vitro* (**Chapter 3**). We recently demonstrated that DmHsp67Bc also shares functional similarity with HSPB8 (not HSPB1) in its capacity to stimulate autophagy (54). This either implies that the protective effect of overexpression of HSPB8 on tachypacing-induced remodeling *in vitro* (**Chapter 4**) is independent of its stimulatory effect on autophagy or that autophagy stimulation is insufficient to provide protection *in vivo*.

Most importantly, the data show that overexpression of small HSPs also protect *in vivo* against AF-induced structural and functional remodeling. This underscores boosting of HSPB expression as a novel therapeutic strategy to ameliorate progression of AF in humans.

5. Exclusive protection against AF by Beat Shock Proteins

HSPB family members do share common features in cell stabilization and survival, however, they are not expressed at equal levels in heart tissue. The HSPB members that show high basal expression in heart comprise of HSPB1, HSPB5, HSPB6, HSPB7 and HSPB8. Interestingly, as shown in **Chapter 3 and Chapter 4,** all of these 'cardiac' HSPB members (with the exception of HSPB5) were found to protect against tachypacing-induced remodeling (Table 1), suggesting basal expression and protective effectiveness to be related. The possible rational behind this may be that their high basal expression level allows these HSPB members to exert an immediate cardioprotective effect in response to stress conditions, without any lag time necessary for protein synthesis. Although HSPB1, HSPB6, HSPB7 and HSPB8 protect against tachypacing-induced remodeling, no general consensus is found with other (protective) effects, such as assisting in protein refolding, reducing aggregation of misfolded proteins and heat inducibility (55) (for overview see Table 1). The findings suggest a unique function shared exclusively by HSPB1, HSPB6, HSPB7 and HSPB8 and therefore we nicknamed this particular group of HSPB members the Beat Shock Proteins (56).

6. Heat shock protein modulation as a novel therapeutic approach in Atrial Fibrillation

So far, the efficacy of commonly used drugs on remodeling is limited, as L-type Ca^{2+} channel blockers, Na^{+}/H^{+} exchange inhibitor and an angiotensin-converting enzyme inhibitor are ineffective in preventing remodeling caused by prolonged (>24 h) periods of atrial tachycardia (57). Drugs with T-type Ca^{2+} channel blocking action, such as

mibefradil (58) and amiodarone (59) prevent atrial tachycardia remodeling, although both also have a wide range of other properties so that the precise mechanism for their beneficial effect is unclear.

Interventions with anti-inflammatory and/or antioxidant compounds, such as glucocorticoids (60) and statins (61), prevent atrial remodeling and may have some efficacy in clinical AF (62,63). Interestingly, these glucocorticoids and statins induce HSPB1 phosphorylation and lead to elevated HSPB1 expression (64,65), leaving open the possibility that the protective effect of these drugs is due to (co-)induction of HSPB1 (Table 2). This supports the search for novel, preferably more specific HSP (co-)inducers as an anti-remodeling intervention in AF. The drug used in our studies, GGA, was originally introduced as an anti-ulcer agent (66) (Table 2). GGA is a non-toxic acyclic isoprenoid compound with a retinoid skeleton that induces HSP synthesis in various tissues, including gastric mucosa, intestine, liver, myocardium, retina, and central nervous system (66,67,68). GGA induces HSP expression by enhancing the activity of heat shock transcription factor HSF1 in cells undergoing (mild) stress (66,69). It is not active in unstressed cells, meaning that it must be considered as a HSF-1 boosting (but not activating) agent (70). This, in fact, may be very advantageous as chronic overexpression of HSP in unstressed cells may have unwanted side-effects (71,72). In our hands, GGA was effective in elevating HSP levels and subsequent AF protection in cell models (**Chapter 3**), Dm Model (**Chapter 5**) as well as in dogs (25). This, in fact, provides sufficient basis for a clinical trial with GGA to prevent AF, albeit that GGA biodistribution remains an issue for improvement. Studies to generate more potent GGA derivatives are therefore currently developed by Nyken BV.

Besides GGA-related compounds, a number of hydroxylamine derivatives such as Bimoclomol and Arimoclomol are known to co-induce the expression of HSPs by prolonging the activation of the heat shock protein transcription factor HSF-1 (73,74) (Table 2). In **Chapter 5,** one of them BGP-15, that already had been shown to protect heart cells from ischemia reperfusion injury (75), was also found to protect against the tachypacing induced reduction in heart rate and loss of heart contraction in *Drosophila.*

Both GGA and BGP15 induced the expression of endogenous DmHSPs (DmHSP70AA and DmHSP27).

Together, our studies clearly reveal the potential of HSP boosting drugs in AF and urge for further testing in the prevention of experimental AF, and ultimately in patients with AF.

7. Summary and Future perspectives

New strategies for the management of AF will depend on a better understanding of the mechanisms underlying AF. Over the past 10 to 15 years, an increased awareness of the role of "atrial remodeling" has substantially advanced the understanding of AF pathophysiology. In brief, the high rate of myocyte activation during AF causes cellular stress and hence activates the atrial remodeling processes, which paradoxically promote persistency of AF. Atrial remodeling is brought about by an interplay of various changes in cellular physiology, which favor (re)-occurrence or maintenance of AF. Further work is needed to more precisely unravel the molecular basis of atrial remodeling, particularly to enable exploitation of their therapeutic potential in the management of AF.

This thesis provides strong indications, both from *in vitro* and *in vivo* models, that HSPs and in particular the beat shock proteins suppress atrial remodeling and the promotion of AF induced by tachyarrhythmia. Utilization of HSPs offers a novel therapeutic strategy in AF, directed at maintaining or restoring tissue integrity and contractile function. Dissection of the molecular mechanism by which HSPs protects myocytes from remodeling may disclose more specific therapeutic interventions. Ultimately, inducers of HSPs may add to the therapeutic armament to delay progression towards persistent AF and/or improve the outcome of cardioversion in patients.

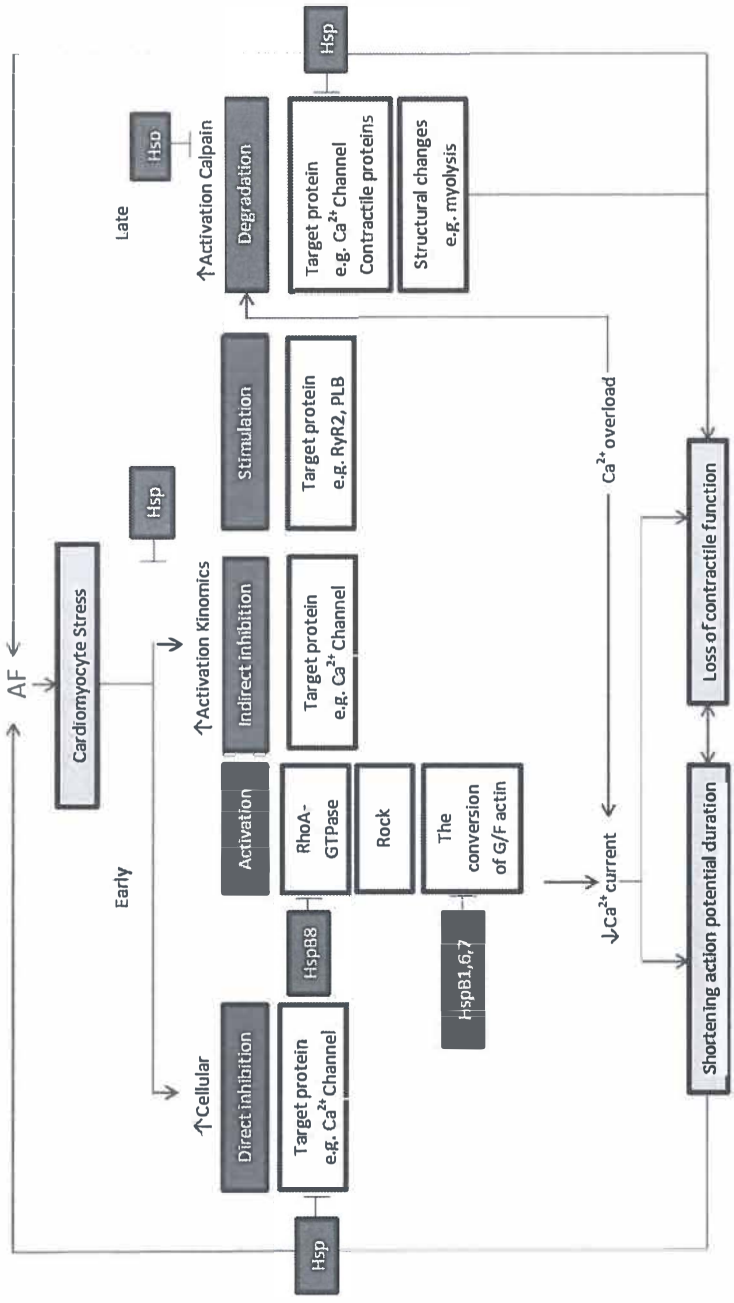


Figure 1 The conceptual model for the mechanisms underlying AF progression and action of small heat shock proteins

Species	Name	Assisting refolding of (heat) denatured proteins	Reducing aggregation of misfolded proteins	Protecting against tachypacing-reduced remodeling	Heat inducibility
Homo sapiens	HSPB1	+++	---	+++	+++
	HSPB2	---	---	---	---
	HSPB3	---	---	---	---
	HSPB4	+++	+--	---	---
	HSPB5	+++	---	---	+++
	HSPB6	+--	+--	+++	---
	HSPB7	---	+++	+++	---
	HSPB8	---	+--	++-	+++
	HSPB9	---	+++	---	---
	HSPB10	---	---	---	---
Drosophila melanogaster	HSP23	+++	+--	+++	+++
	HSP26	+++	+--	???	+++
	HSP27	+++	---	---	+++
	HSP67BA	---	---	???	++-
	HSP67BC	---	+++	---	+++
	L2EFL	+--	---	???	+++
	CG4461	---	---	---	+++
	CG7409	+++	+--	---	++-
	CG13133	---	---	???	++-
	CG14207	+++	---	---	---

Table 1 Summarized characteristics and activities for human and Drosophila small HSPs

Compound	Indications	Target
Geranylgeranhylacetone	Atrial fibrillation	HspB1
	Ischaemic heart diseases	HspA1A
Dexamethasone	Ischaemic heart diseases	HSF-1
Atorvastatin	Ischaemic heart diseases	HspB1-Phosphorylation
Bimoclomol	Ischaemic heart diseases	HspA1A
Cyclosporine A	Ischaemic heart diseases	HspA1A

Table 2 HSP inducing compounds for the heart (76)

References

1. Nattel S. New ideas about atrial fibrillation 50 years on. *Nature* **2002**;415:219 - 226.

2. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation.* **1995**;92(7):1954-1968.

3. Van Gelder IC, Crijns HJ, Tieleman RG, Brügemann J, De Kam PJ, Gosselink AT, *et al*. Chronic atrial fibrillation. Success of serial cardioversion therapy and safety of oral anticoagulation. *Arch Intern Med.* **1996**;156(22):2585-2592.

4. Todd DM, Fynn SP, Walden AP, Hobbs WJ, Arya S, Garratt CJ. Repetitive 4-week periods of atrial electrical remodeling promote stability of atrial fibrillation: time course of a second factor involved in the self-perpetuation of atrial fibrillation. *Circulation.* **2004**;109(11):1434-1439.

5. Sun H, Gaspo R, Leblanc N, Nattel S. Cellular mechanisms of atrial contractile dysfunction caused by sustained atrial tachycardia. *Circulation.* **1998**;98(7):719-727.

6. Yue L, Melnyk P, Gaspo R, Wang Z, Nattel S. Molecular mechanisms underlying ionic remodeling in a dog model of atrial fibrillation. *Circ Res.* **1999**;84(7):776-784.

7. Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. Circ Res. **2006**;98(6):730-742.

8. Qi XY, Yeh Y, Xiao L, Nattel S. Molecular mechanisms of tachycardia-dependent atrial remodeling probed in a novel in vitro model. *Circulation.* **2006**;114(Suppl. 11):11-292.

9. Christ T, Boknik P, Wöhrl S, Wettwer E, Graf EM, Bosch RF, *et al*. L-type Ca2+ current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases. *Circulation.* **2004**;110(17):2651-2657.

10. Greiser M, Halaszovich CR, Frechen D, Boknik KP, Ravens U, Dobrev D *et al*. Pharmacological evidence for altered src kinase regulation of I (Ca,L) in patients with chronic atrial fibrillation. *Naunyn Schmiedebergs Arch Pharmacol.* **2007**;375(6):383 - 392.

11. El-Armouche A, Boknik P, Eschenhagen T, Carnier L, Knaut M, Ravens U *et al*. Molecular determinants of altered Ca2t handling in human chronic atrial fibrillation. *Circulation.* **2006**;114(7):670 - 680.

12. Vest JA, Wehrens XHT, Reiken SR, Lehnart SE, Dobrev D, Chandra P et al. Defective cardiac ryanodine receptor regulation during atrial fibrillation. *Circulation.* **2005**;111(16):2025 - 2032.

13. Brundel BJ, Ausma J, van Gelder IC, Van der Want JJ, van Gilst WH, Crijns HJ, Henning RH. Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res.* **2002**;54(2):380-389.

14. Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, and Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation*, **1997**;96(9):3157-3163.

15. Sherman AJ, Klocke FJ, Decker RS, Decker ML, Kozlowski KA, Harris KR, Hedjbeli S, Yaroshenko Y, Nakamura S, Parker MA, Checchia PA, and Evans DB. Myofibrillar disruption in hypocontractile myocardium showing perfusion-contraction matches and mismatches. *Am J Physiol Heart Circ Physiol.* **2000**;278(4):1320-1334.

16. Vanoverschelde JL, Wijns W, Depré C, Essamri B, Heyndricks S, Borgers M, Bol A, and Melin J. Mechanisms of chronic regional postischemic dysfunction in humans: new insights from the study on

non-infarcted collateral dependent myocardium. *Circulation.* **1993**;87(5):1513-1523.

17. Bito V, Heinzel FR, Weidemann F, Dommke C, Van Der Velden J, Verbeken E, Claus P, Bijnens B, De Scheerder I, Stienen GJM, Sutherland GR, and Sipido KR. Cellular mechanisms of contractile dysfunction in hibernating myocardium. *Circ Res.* **2004**;94(6):794-801.

18. Leist M, Jäättelä M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol.* **2001**;2(8):589-598.

19. Kepplinger KJ, Forstner G, Kahr H, Leitner K, Pammer P, Groschner K *et al.* Molecular determinant for run-down of L-type Ca2+ channels localized in the carboxyl terminus of the 1C subunit. *J Physiol.* **2000**;529 Pt 1:119-130.

20. Belles B, Hescheler J, Trautwein W, Blomgren K, Karlsson JO. A possible physiological role of the Ca-dependent protease calpain and its inhibitor calpastatin on the Ca current in guinea pig myocytes. *Pflugers Arch.* **1988**;412(5):554-556.

21. Moretti A, Weig HJ, Ott T, Seyfarth M, Holthoff HP, Grewe D *et al.* Essential myosin light chain as a target for caspase-3 in failing myocardium. *Proc Natl Acad Sci USA.* **2002**;99(18):11860-11865.

22. Communal C, Sumandea M, de Tombe P, Narula J, Solaro RJ, Hajjar RJ. Functional consequences of caspase activation in cardiac myocytes. *Proc Natl Acad Sci USA.* **2002**;99(9):6252-6256.

23. Zhang Z, Biesiadecki BJ, Jin JP. Selective deletion of the NH2-terminal variable region of cardiac troponin T in ischemia reperfusion by myofibril-associated mu-calpain cleavage. *Biochemistry.* **2006**;45(38):11681-11694.

24. Andrea Natale and José Jalife. Atrial Fibrillation: From Bench to Bedside. Totowa, NJ, Humana Press, **2008**.

25. Brundel BJ, Shiroshita-Takeshita A, Qi X, Yeh YH, Chartier D, van Gelder IC, *et al.* Induction of heat shock response protects the heart against atrial fibrillation. *Circ Res.* **2006**;99(12):1394-1402.

26. Eric C. R. Reeve, ed (**2001-06-23**). "Drosophila melanogaster: The Fruit Fly". Encyclopedia of genetics. USA: Fitzroy Dearborn Publishers, I. pp. 157.

27. Nishimura M, Ocorr K, Bodmer R, Cartry J. Drosophila as a model to study cardiac aging. *Exp Gerontol.* **2011**;46(5):326-330.

28. Ocorr KA, Crawley T, Gibson G, Bodmer R. Genetic variation for cardiac dysfunction in Drosophila. *PLoS One.* **2007**;2(7):e601.

29. Tickoo S, Russell S. Drosophila melanogaster as a model system for drug discovery and pathway screening. *Curr Opin Pharmacol.* **2002**;2(5):555-560.

30. Johnson E, Sherry T, Ringo J, Dowse H. Modulation of the cardiac pacemaker of Drosophila: cellular mechanisms. *J Comp Physiol B.* **2002**;172(3):227-236.

31. Sõti C, Nagy E, Giricz Z, Vígh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol.* **2005**;146(6):769-780.

32. Yang M, Tan H, Cheng L, He M, Wei Q, Tanguay RM, *et al.* Expression of heat shock proteins in myocardium of patients with atrial fibrillation. *Cell Stress Chaperones.* **2007**;12(2):142-150.

33. Brundel BJ, Kampinga HH, Henning RH. Calpain inhibition prevents pacing-induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. *Cardiovasc Res.* **2004**;62(3):521-528.

34. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol.* **1995**;15(1):505-516.

35. Bryantsev AL, Loktionova SA, Ilyinskaya OP, Tararak EM, Kampinga HH, Kabakov AE. Distribution, phosphorylation, and activities of Hsp25 in heat-stressed H9c2 myoblasts: a functional link to cytoprotection. *Cell Stress Chaperones.* **2002**;7(2):146-155.

36. Somara S, Bitar KN. Tropomyosin interacts with phosphorylated HSP27 in agonist-induced contraction of smooth muscle. *Am J Physiol Cell Physiol.* **2004**;286(6):C1290-1301.

37. Venkatakrishnan CD, Tewari AK, Moldovan L, Cardounel AJ, Zweier JL, Kuppusamy P, et al. Heat shock protects cardiac cells from doxorubicin-induced toxicity by activating p38 MAPK and phosphorylation of small heat shock protein 27. *Am J Physiol Heart Circ Physiol.* **2006**;291(6):H2680-2691.

38. Leach RN, Desai JC, Orchard CH. Effect of cytoskeleton disruptors on L-type Ca channel distribution in rat ventricular myocytes. *Cell Calcium.* **2005**;38(5):515-526.

39. Armstead WM, Hecker JG. Heat shock protein modulation of KATP and KCa channel cerebrovasodilation after brain injury. *Am J Physiol Heart Circ Physiol.* **2005**;289(3):H1184-1190.

40. Ficker E, Dennis AT, Wang L, Brown AM. Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel HERG. *Circ Res.* **2003**;92(12):e87-100.

41. Krieger A, Radhakrishnan K, Pereverzev A, Siapich SA, Banat M, Kamp MA, *et al.* The molecular chaperone hsp70 interacts with the cytosolic II-III loop of the Cav2.3 E-type voltage-gated Ca2+ channel. *Cell Physiol Biochem.* **2006**;17(3-4):97-110.

42. Kashlan OB, Mueller GM, Qamar MZ, Poland PA, Ahner A, Rubenstein RC, *et al.* Small heat shock protein alphaA-crystallin regulates epithelial sodium channel expression. *J Biol Chem.* **2007**;282(38):28149-28156.

43. Patil SB, Pawar MD, Bitar KN. Phosphorylated HSP27 essential for acetylcholine-induced association of RhoA with PKCalpha. *Am J Physiol Gastrointest Liver Physiol.* **2004**;286(4):G635-644.

44. Patil SB, Pawar MD, Bitar KN. Direct association and translocation of PKC-alpha with calponin. *Am J Physiol Gastrointest Liver Physiol.* **2004**;286(6):G954-963.

45. Park KJ, Gaynor RB, Kwak YT. Heat shock protein 27 association with the I kappa B kinase complex regulates tumor necrosis factor alpha-induced NF-kappa B activation. *J Biol Chem.* **2003**;278(37):35272-35278.

46. Kammanadiminti SJ, Chadee K. Suppression of NF-kappaB activation by Entamoeba histolytica in intestinal epithelial cells is mediated by heat shock protein 27. *J Biol Chem.* **2006**;281(36):26112-26120.

47. Cardin S, Li D, Thorin-Trescases N, Leung TK, Thorin E, Nattel S. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways. *Cardiovasc Res.* **2003**;60(2):315-325.

48. Li D, Shinagawa K, Pang L, Leung TK, Cardin S, Wang Z, *et al.* Effects of angiotensin-converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing-induced congestive heart failure. *Circulation.* **2001**;104(21):2608-2614.

49. Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, Doseff AI. Binding of caspase-3

prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J Biol Chem.* **2007**;282(34):25088-25099.

50. Michaud S, Marin R, Tanguay RM. Regulation of heat shock gene induction and expression during Drosophila development. *Cell Mol Life Sci.* **1997**;53(1):104-113.

51. Vos MJ. Small heat shock proteins: implication for neurodegeneration and longevity. *Thsis.* **2009**.

52. Morrow G, Heikkila JJ, Tanguay RM. Differences in the chaperone-like activities of the four main small heat shock proteins of Drosophila melanogaster. *Cell Stress Chaperones.* **2006**;11(1):51-60.

53. Vos MJ, Hageman J, Carra S, Kampinga HH. Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. *Biochemistry.* **2008**;47(27):7001-7011.

54. Carra S, Boncoraglio A, Kanon B, Brunsting JF, Minoia M, Rana A, et al. Identification of the Drosophila ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J Biol Chem.* **2010**;285(48):37811-37822.

55. Vos MJ, Zijlstra MP, Carra S, Sibon OC, Kampinga HH. Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy.* **2011**;7(1):101-103.

56. Kampinga HH, Henning RH, van Gelder IC, Brundel BJ. Beat shock proteins and atrial fibrillation. *Cell Stress Chaperones.* **2007**;12(2):97-100.

57. Shinagawa K, Derakhchan K, Nattel S. Pharmacological prevention of atrial tachycardia induced atrial remodeling as a potential therapeutic strategy. *Pacing Clin Electrophysiol.* **2003**;26(3):752-764.

58. Fareh S, Bénardeau A, Thibault B, Nattel S. The T-type Ca(2+) channel blocker mibefradil prevents the development of a substrate for atrial fibrillation by tachycardia-induced atrial remodeling in dogs. *Circulation.* **1999**;100(21):2191-2197.

59. Shinagawa K, Shiroshita-Takeshita A, Schram G, Nattel S. Effects of antiarrhythmic drugs on fibrillation in the remodeled atrium: insights into the mechanism of the superior efficacy of amiodarone. *Circulation.* **2003**;107(10):1440-1446.

60. Shiroshita-Takeshita A, Brundel BJ, Lavoie J, Nattel S. Prednisone prevents atrial fibrillation promotion by atrial tachycardia remodeling in dogs. *Cardiovasc Res.* **2006**;69(4):865-875.

61. Shiroshita-Takeshita A, Schram G, Lavoie J, Nattel S. Effect of simvastatin and antioxidant vitamins on atrial fibrillation promotion by atrial-tachycardia remodeling in dogs. *Circulation.* **2004**;110(16):2313-2319.

62. Dernellis J, Panaretou M. Relationship between C-reactive protein concentrations during glucocorticoid therapy and recurrent atrial fibrillation. *Eur Heart J.* **2004**;25(13):1100-1107.

63. Ozaydin M, Varol E, Aslan SM, Kucuktepe Z, Dogan A, Ozturk M, *et al.* Effect of atorvastatin on the recurrence rates of atrial fibrillation after electrical cardioversion. *Am J Cardiol.* **2006**;97(10):1490-1493.

64. Nègre-Aminou P, van Leeuwen RE, van Thiel GC, van den IJssel P, de Jong WW, Quinlan RA *et al.* Differential effect of simvastatin on activation of Rac(1) vs. activation of the heat shock protein 27-mediated pathway upon oxidative stress, in human smooth muscle cells. *Biochem Pharmacol.* **2002**;64(10):1483-1491.

65. Son GH, Geum D, Chung S, Park E, Lee KH, Choi S, Kim K. A protective role of 27-kDa heat shock protein in glucocorticoid-evoked apoptotic cell death of hippocampal progenitor cells. *Biochem Biophys Res Commun.* **2005**;338(4):1751-1758.

66. Hirakawa T, Rokutan K, Nikawa T, Kishi K. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology.* **1996**;111(2):345-357.

67. Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, et al. Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. *Circulation.* **2001**;104(15):1837-1843.

68. Katsuno M, Sang C, Adachi H, Minamiyama M, Waza M, Tanaka F, et al. Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. *Proc Natl Acad Sci USA.* **2005**;102(46):16801-16806.

69. Yamanaka K, Takahashi N, Ooie T, Kaneda K, Yoshimatsu H, Saikawa T. Role of protein kinase C in geranylgeranylacetone-induced expression of heat-shock protein 72 and cardioprotection in the rat heart. *J Mol Cell Cardiol.* **2003**;35(7):785-794.

70. Yamagami K, Yamamoto Y, Ishikawa Y., Yonezawa K, Toyokuni S, Yamaoka Y. Effects of geranyl-geranyl-acetone administration before heat shock preconditioning for conferring tolerance against ischemia-reperfusion injury in rat livers. *J Lab Clin Med.* **2000**;135(6):465-475.

71. Khalil AA, Kabapy NF, Deraz SF, Smith C. Heat shock proteins in oncology: Diagnostic biomarkers or therapeutic targets? *Biochim Biophys Acta.* **2011**;1816(2):89-104.

72. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell.* **2007**;130(6):1005-1018.

73. Hargitai J, Lewis H, Boros I, Rácz T, Fiser A, Kurucz I, *et al.* Bimoclomol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1. *Biochem Biophys Res Commun.* **2003**;307(3):689-695.

74. Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L. Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. *Nat Med.* **2004**;10(4):402-405.

75. Szabados E, Literati-Nagy P, Farkas B, Sumegi B. BGP-15, a nicotinic amidoxime derivate protecting heart from ischemia reperfusion injury through modulation of poly(ADP-ribose) polymerase. *Biochem Pharmacol.* **2000**;59(8):937-945.

76. Brundel BJ, Ke L, Dijkhuis AJ, Qi X, Shiroshita-Takeshita A, Nattel S, *et al.* Heat shock proteins as molecular targets for intervention in atrial fibrillation. *Cardiovasc Res.* **2008**;78(3):422-428.

Nederlandse Samenvatting

Boezemfibrilleren is een hardnekkige hartritmestoornis, die leidt tot een sterke toename van het aantal samentrekkingen per tijdseenheid in de boezem spiercellen (cardiomyocyten). De ritmestoornis gaat gepaard met een duidelijk verhoogde kans op het ontwikkelen van andere (hart)ziekten. Verder is boezemfibrilleren een progressieve aandoening, waardoor veel patiënten die beginnen met af en toe periodes met boezemfibrilleren (paroxysmaal boezemfibrilleren) later continu, chronisch boezemfibrilleren zullen krijgen. Daarnaast is de kans op een succesvolle cardioversie, het weer terug brengen in het normale hartritme, in patiënten met chronisch boezemfibrilleren kleiner dan in paroxysmaal boezemfibrilleren. Dit komt doordat de normale functie van de spiercellen in de boezem na cardioversie zich niet hersteld. De onderliggende oorzaak hiervan zijn veranderingen in de elektrische en structurele opbouw van de spiercellen (cardiomyocyt). Al deze veranderingen samen noemen we cardiomyocyt remodellering.

Wat gebeurd er precies als boezemfibrilleren ontstaat? Door de verhoogde elektrische activatie van de boezem neemt de cardiomyocyt calcium op in de cel via het L-type calcium kanaal. Het verhoogde calcium niveau in de cardiomyocyt is schadelijk en de myocyt reageert hierop door de stroom door het L-type calcium kanaal te verminderen, waardoor er elektrische veranderingen ontstaan. Daarnaast zorgt de toename in calcium ervoor dat er eiwitafbrekende enzymen (proteases) geactiveerd worden. We hebben in het verleden al aangetoond dat patiënten met chronisch boezemfibrilleren meer actief calpaïne (een calcium-gereguleerde protease) in de cardiomyocyt hebben. Het verhoogde calpaïne zou in patiënten ervoor kunnen zorgen dat de spiereiwitten worden afgebroken, waardoor structurele remodellering en stoornissen in het samentrekken van de cardiomyocyt ontstaat. In **hoofdstuk 2** wordt aangetoond dat dit inderdaad gebeurd in ons *in vitro* boezemmyocyten model voor boezemfibrilleren. Er vindt afbraak plaats van eiwitten betrokken bij de samentrekking (contractie) van cardiomyocyten: de cardiale troponines C, I en T. Door het boezemfibrilleren worden deze eiwitten afgebroken en remmers van calpaïne voorkomen deze afbraak. Remmers die werken op deze troponines, konden deze afbraak en contractiele dysfunction verhinderen terwijl remmers van andere proteases (het proteasoom of caspases) niet werkzaam waren. In patiënten met chronisch boezemfibrilleren is ook eenzelfde afname in cardiale troponines te zien, en deze afname correleert met een toename in calpaïne activiteit. Deze bevindingen geven aan dat boezemfibrilleren calpaïnes activeren, die zorgen voor de afbraak van contraciele eiwitten, cardiale troponines. Vermindering in contractiele eiwitten kan weer leiden tot de hardnekkigheid van boezemfibrilleren.

Doordat boezemfibrilleren een stress reactie veroorzaakt in de boezemmyocyt, hebben we in **hoofdstuk 3** gekeken of er ook beschermende eiwitten tot expressie worden gebracht in boezemfibrilleren. Een belangrijke klasse beschermende eiwitten zijn de zogenaamde heat shock proteïnen (HSP). HSP helpen in de vouwing van nieuwe eiwitten, maar beschermen ook de door stress gedeeltelijk ontvouwde eiwitten. HSP vormen een centraal onderdeel van het eiwitkwaliteitscontrole systeem van de cel, dat zorg draagt voor een juiste eiwithomeostase, ook wel proteostase genoemd. We hebben

gevonden dat patiënten met paroxysmaal boezemfibrilleren een verhoogde expressie hebben van HSP27 (HSPB1) en deze verhoogde expressie neemt af naarmate de patiënt langer chronisch boezemfibrilleren heeft. Deze bevindingen suggereren dat boezemfibrilleren initieel een beschermende HSP reactie geven maar dat door uitputting van de HSP reactie boezemfibrilleren chronischer en hardnekkiger wordt. Inductie van HSP expressie kan dan beschermend werken, zoals we eerder aantoonden voor HSPB1. Andere HSPs, zoals HSP70 (HSPA1A), hadden geen beschermende effecten.

Omdat HSPB1 een eiwit is uit de familie van kleine HSPs, hebben we onderzocht of andere leden van deze familie eenzelfde beschermend effect hebben (**hoofdstuk 4**). In het *in vitro* model voor boezemfibrilleren bleek dat naast HSPB1, ook HSPB6, HSPB7 en HSPB8 een beschermend effect hebben. Omdat al deze HSPs binden aan het netwerk van actine, lijkt het erop dat hun beschermende effect in boezemfibrilleren te wijten is aan het voorkomen van veranderingen in dit netwerk. Actine speelt een belangrijke rol in de structuur en contractiele functie van de cardiomyocyt. Vanuit de literatuur is ook bekend dat RhoA-GTPases het netwerk van actine kan veranderen. Om te onderzoeken of dat ook in ons model het geval is hebben wij eerst de activiteit van RhoA GTPases gemeten tijdens boezemfibrilleren. We vonden een stapsgewijze toename in actief RhoA-GTPase tijdens boezemfibrilleren, die gepaard ging met de vorming van zogenaamde actine stress bundels en afname van contractiele functie. Overexpressie van HSPB1, HSPB6, HSPB7 en HSPB8 verminderde het aantal stress bundels. Bovendien suggereren experimenten met geisoleerd actine dat HSPB1, HSPB6 en HSPB7 dit doen door directe binding aan het actine. HSPB8 daarentegen remde de stress bundel formatie door de activiteit van RhA-GTPase te verminderen.

Bovengenoemde resultaten zijn verkregen gebruikmakend van een in vitro celmodel voor boezemfibrilleren. Om de vertaalslag te maken naar de *in vivo* situatie hebben wij een uniek fruitvlieg model voor boezemfibrilleren ontwikkeld (**hoofdstuk 5**). Een belangrijk voordeel van de fruitvlieg is de mogelijk tot genetisch manipuleren, snelheid van het opkweken van vliegen en de lage kosten. Wij hebben het hart van transparante larven van de fruitvlieg onderworpen aan snelle elektrische stimulaties. De larven ontwikkelen dan contractiele dysfunctie en hebben een verhoogde kans op ritmestoornissen. Ook vonden we structurele remodellering in de vorm van afbraak van contractiele eiwitten en activatie van calpaïnes. Wanneer de larven voorbehandeld werden met HSP-inducerende geneesmiddelen, traden al deze remodelleringen niet op. Vervolgens hebben we transgene vliegen gekweekt die specifieke eiwitten uit de Drosophila (Dm) familie van kleine HSP tot expressie brachten in het hart. Van al de kleine HSP was alleen DmHSP23 beschermend tegen remodellering. DmHSP23 is waarschijnlijk een functionele ortholoog van humaan HSP27. Het onderzoek in de fruitvlieg toont aan dat de fruitvlieg een uitstekend modelsysteem is voor onderzoek naar remodellering geïnduceerd door boezemfibrilleren. Dit systeem biedt vele mogelijkheden om het expressieniveau van allerlei eiwitten te manipuleren om zo hun effect op remodellering te bestuderen.

Samengevat toont het onderzoek dat remodellering van boezemspiercellen tijdens boezemfibrilleren verloopt via de activatie van RhoA-GTPases, stress bundel formatie en calpaïne activatie. Deze schadeprocessen kunnen worden voorkomen door de inductie

van HSP, waarbij specifieke leden van de kleine HSP familie erg effectief zijn. Dezelfde resultaten werden behaald in de gestimuleerde fruitvlieglarve. Enerzijds is daarmee een nuttig, nieuw modelsysteem voor atriumfibrilleren ontwikkeld; anderzijds toont het de brede toepassing van het HSP systeem in remodelering van het hart aan. Bovendien suggereren de resultaten dat nieuwe geneesmiddelen die zorgen voor de inductie van HSPs, zoals bijvoorbeeld geranylgeranylacetone (GGA), klinisch erg bruikbaar kunnen zijn door het behoud van boezemfunctie na cardioversie. Daarnaast kan de opgedane kennis ook gebruikt worden voor andere hartaandoeningen die ook gepaard gaan met (ernstige) structurele remodellering.

Acknowledgements

As I am finally approaching the completion of this thesis and reflecting on the past years, I can't help but think how memorable the journey it has been. This experience makes me realize where I am today. To all of you who have made it possible, thank you!

Firstly, I would like to deeply thank my promoters, Prof. Harm H. Kampinga and Prof. Robert H. Henning for having me into this PhD project. It is an absolute pleasure having this excellent opportunity to learn and develop a range of transferable skills that also help guide me in my current career. I am also very grateful to both of you for your scientific advice, knowledge, insightful discussion and suggestions.

It is difficult to put into words the gratitude that is owed to my supervisor Bianca. Without you, the work would have never been completed. During the most difficult times when writing this thesis, especially after leaving Groningen, your advice and assistance on experiments, proof reading my manuscripts, continued encouragement and faith in my ability have pushed me to achieve goals of which I did not believe I was capable.

My gratitude is also extended to the members of the reading committee, Prof. dr. U. Schotten, Prof. dr. R. Tanguay and Prof. dr. I.C. van Gelder for evaluating my thesis.

Finding a way to settle down in a new place is not easy. The things, like IND, finding a proper health insurance, filing tax return, dealing with lengthy documents written in Dutch etc, are quite challenging for newcomers to the "low land". Many thanks to Annet, Gerry, Rita, Ms.Riekje Banus and Ms.Anneke Toxopeus. You have been of a great help to me when I was in Groningen. You have done a lot of favors to me and made my stay in Groningen comfortable and useful.

Completing and Publishing all the work would have been all the more difficult were it not for the support and involvement provided by Anne-Jan, Deli, Joris, Roelien and Xiaoyan Qi. Thank you all for help and contribution. My warm thanks are also due to Klaas Sjollema in Molecular Imaging and Electron Microscopy for helping out with live imaging work. Your expertise and kindness are sincerely appreciated.

Many thanks to Prof. Ody Sibon and Dr. Rob Coppes for your critical inputs on this project. Your efforts were much appreciated. I am grateful for other colleagues in Radiation and Stress Cell Biology: Anil, Bart, Erwin, Floris, Hette, Isabelle, Jeanette, Jurre, Jielin Feng, Karin, Kasia, Maarten, Maria, Maria, Marianne, Martha, Michel, Serena, Willy, Xia Yi. You have contributed immensely to my professional and personal time at UMCG. You have been a source of friendships as well as good advice and collaboration. I would like to extend my thanks to Prof. Gerald de Haan and my

colleagues in Stem Cell Biology for all your help.

My life in the "low land" would have never been so memorable without a group of friends who speak the same language and know the same culture: Wu Bian and Qiqi, Xie Hangxing and Geng Ying, Mei Li and Qu Wenwen, Qian Cheng and Yu Lili, Wang Ying and Zhou Lu, Ou Jianghua and your wife, Yu Hongyou and Feng Jieling, Qi Liqiang and your wife, Han Lina and Zhao Yan, Jing Jianfeng and Qin Lin, Yu Jingyan, Ding Ning, Fu Jingyuan, He Tao, Wang Hongwei, Ma Yue, Ye Qingshong, Xu Chunping, Xu Chuanhui, Tan Hongtao, Tan Lijun, Yi Xia, Yang Nan, Li Xiangyi, Li Yang and many others. We have done a lot of interesting things together: driving, cycling, skiing stunts, holidays across European countries, dinners, parties, nights out, games, lunchtime at UMCG canteens…. Good time with you all is unforgettable and may this continue wherever we meet each other again in the world.

To my friends Yang Qing, Yan Qingbo, Chen Yi, Wang Jing, Guo Dejun: thank you for all your continuous support from a distance. A big thank you goes to Prof. dr. Andrew Sandham. Your kind-heartedness shines through in many ways: your encouragement, insightful suggestions, tremendous help and lots of others. I am delighted to meet and have you as my mentor.

To Xialing: Through your understanding, support, love and belief in me, I have been able to complete this long journey. You gave me many precious memories and made a place in my heart only you will ever occupy. We have laughed and cried, traveled and hanged out, built and settled, and planned and discussed our lives. When I went through difficulties and mood change, you were patient with me and waited for me to think it through. You have given me confidence and motivated me in so many ways. There are no words that can express my gratitude and appreciation for all you have done and been for me.

亲爱的爸爸妈妈：在外这么多年，每当我遇到困惑、困难和挫折的时候，您们总是无私地以各种形式支持我，激励我不断前进。在这里对您们致以我深深的感谢，感谢您们一直以来对我的宽容、理解和为我付出的一切！

With Love